Pillar® Biosciences PiVAT® User Manual

Software Version 2023.1.0

Released June 2023

Legal Notice

For Research Use Only. Not for use in diagnostic procedures.

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Introduction

This user manual describes how to use Pillar Bioscience's (Pillar) PiVAT software. PiVAT is Pillar's genome sequence data software.

Software Requirements

The PiVAT software requires a supported browser to access the user interface. The current versions of the following browsers are supported:

- Google Chrome
- Mozilla Firefox

Symbols Used in this User Manual:

This symbol is a reminder to pay attention to details that could affect either proper installation or performance**.**

This symbol is an indication of a critical detail not to be overlooked.

Connect to PiVAT

1. Login to PiVAT using the username and password provided by Pillar Biosciences.

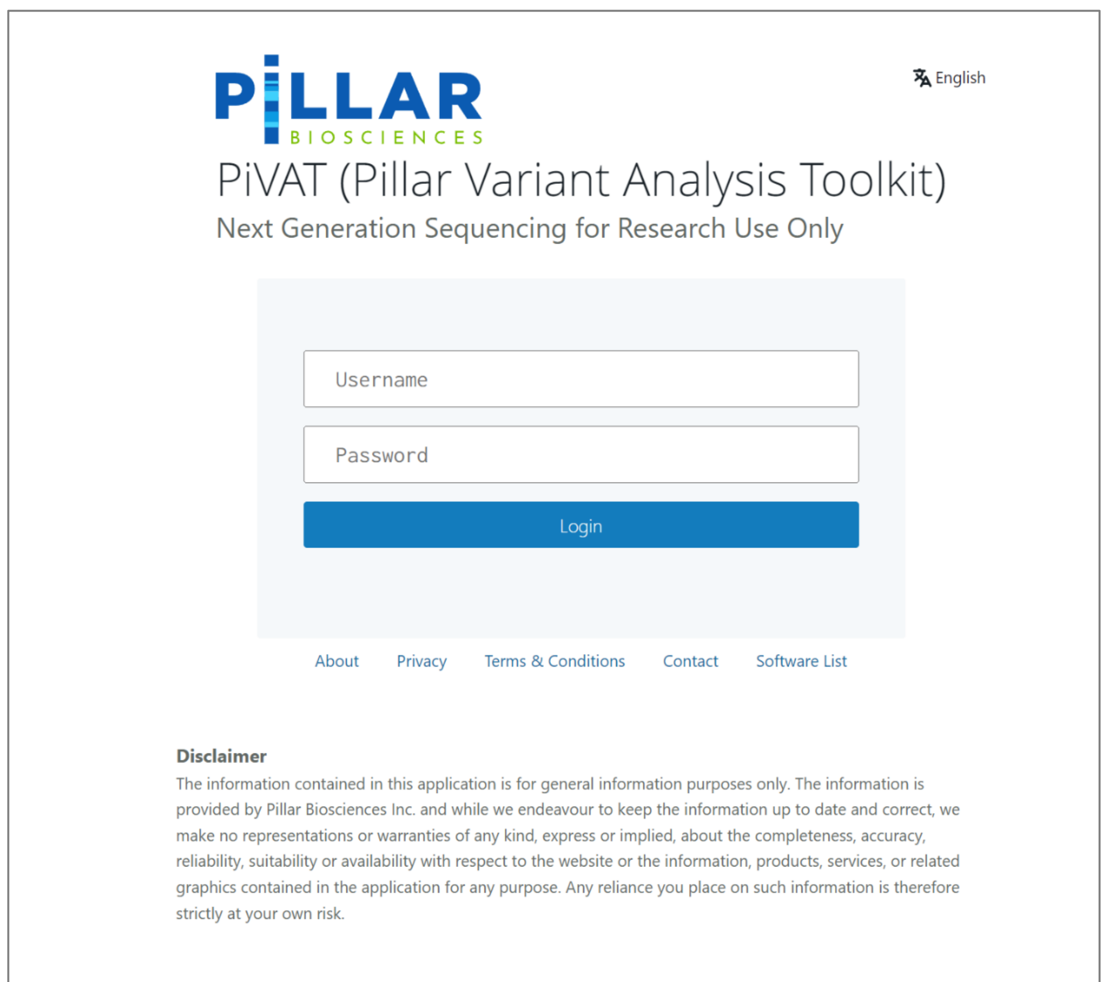


Figure 1 PiVAT login page

Accept Terms and Conditions

1. The following screen is displayed upon user's first login.
2. Read the terms and conditions and click the **Agree** button to continue.

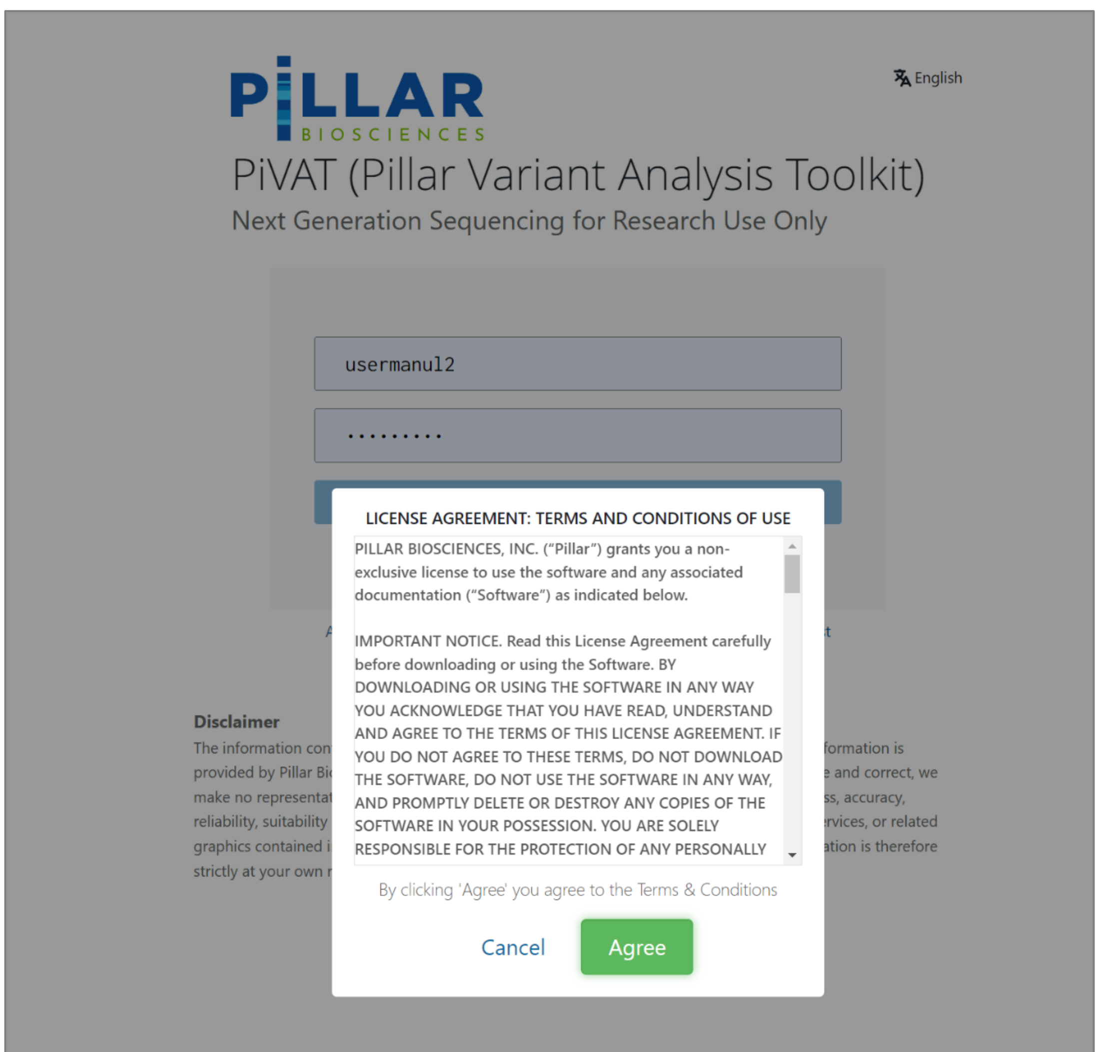


Figure 2 PiVAT License Agreement

Using the PiVAT Software

Dashboard

1. User may navigate through the application using the Dashboard screen.

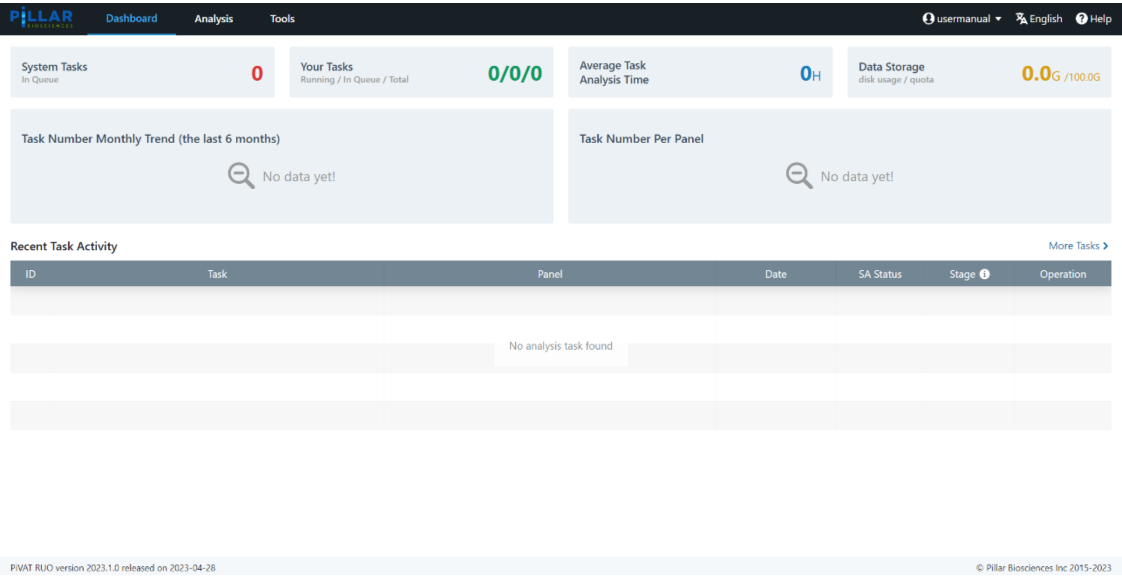


Figure 3 PiVAT Dashboard page

Navigation Bar

1. The following Navigation Bar is displayed on the top of PiVAT Dashboard page. Additional items are displayed for admin users and detailed in the PiVAT Admin Guide document.
2. The page being displayed will be highlighted on the Navigation Bar.
3. Logged in user is indicated next to 👤 icon.
4. PiVAT software is enabled for English and Chinese languages.

Figure 4 Navigation Bar on PiVAT Dashboard

Dashboard: Returns the user to the Dashboard screen

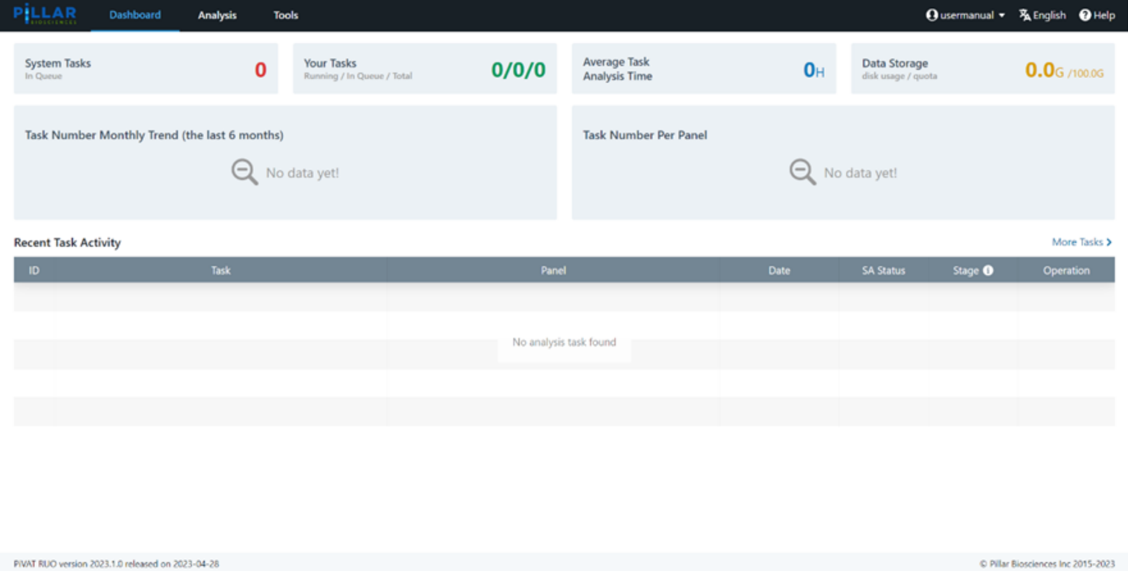


Figure 5 PiVAT Dashboard page

Analysis: Displays the screen for creating, starting, and viewing analysis tasks on the left menu bar include:

- **Parameters**: Used to create a custom set of secondary analysis parameters for running analysis (optional section for advanced users).
- **Start Analysis**: Most users will start here after uploading data in the Data Management section.
- **Analysis Results**: Detailed view of queued and completed analysis tasks.

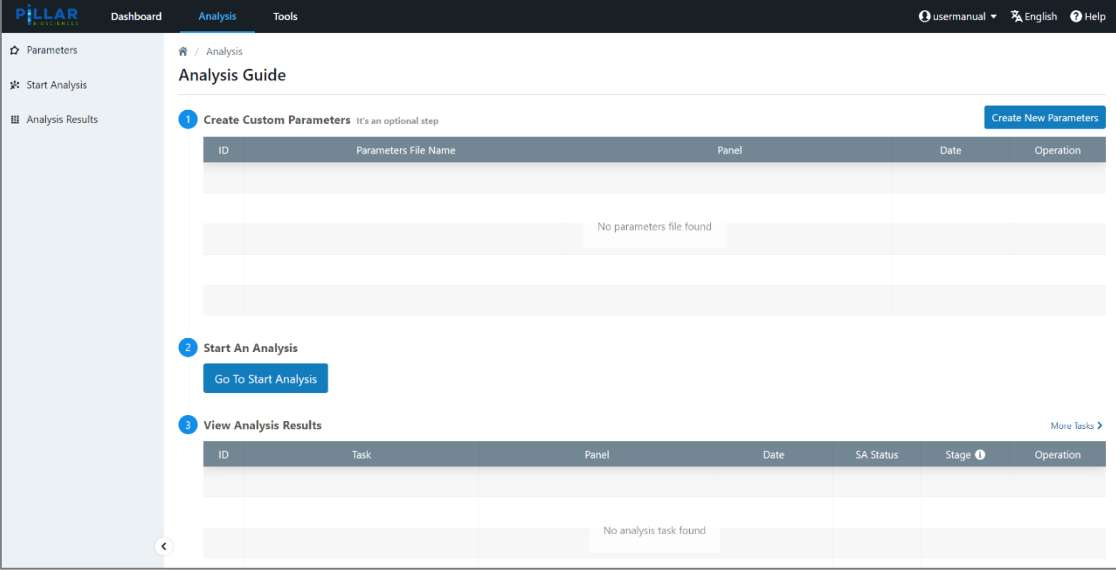


Figure 6 PiVAT Analysis page

Tools: Displays the screens for Integrative Genomics Viewer (IGV), Data Management and Download on the left menu bar.

- **IGV**: Choose *bam* and *bai* files from task server, and enter the locus you want to focus on.
- **Data Management**: Sample data files can be uploaded to this section for use as inputs to the analysis tasks. The Project Name corresponds to a folder with the same name in the uploads directory. Sample files used for a task can hence be uploaded to a Project and be used as a bundled input for the Analysis Task.
- **Download**: Select and download panel and analysis parameters in bed format.

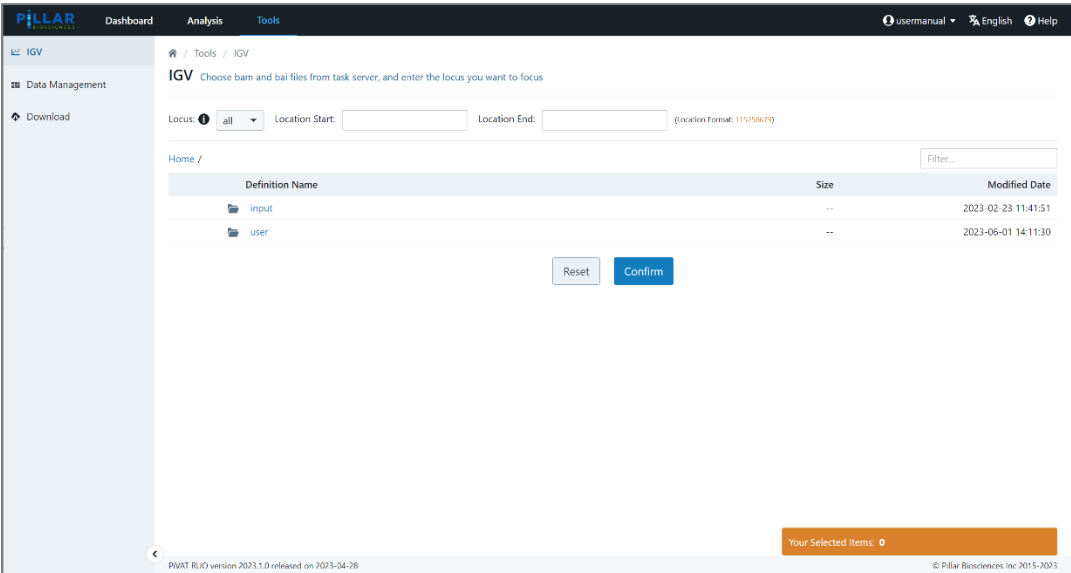


Figure 7 PiVAT Tools page

Help: Displays product information, instructions for SFTP, and a downloadable User Manual in PDF format .

Help

Help Retrieve Product Documentation

Versions

```
Pivat 2023.1.0
Analysis Pipeline 2023.1.0
```

PiVAT User Manual

*If you want to learn about how to use this software, please download our 'User Manual'.

SFTP Download Instructions

To download files using SFTP (Secure File Transfer Protocol), your installation of PiVAT must have the SFTP feature enabled by the administrator. Please contact your administrator to confirm if this feature is available. To use the SFTP feature, SFTP client software is required such as FileZilla or Cyberduck. Use the following settings.

Address: sftp://gt.pillar-biosciences.net
Port: 2225
Username: Your PiVAT username
Password: Your PiVAT password

Notes:
* When uploading is interrupted, the file number and total size may be incorrect, please logout & login to the SFTP client again so the system can refresh. Deletion of corrupted files will also require you to logout and login again.
* Some SFTP clients, FileZilla for example, may run into an infinite loop of retrying an upload when a file exceeds the size limitation. Please terminate it manually.

Software List

Package	Version	License	Link
BWA	0.7.16a-r1181	Apache 2.0	https://github.com/lh3/bwa
VEP	106.1	Apache 2.0	https://github.com/Ensembl/ensembl-vep
samtools	1.10	The MIT/Expat License	https://github.com/samtools/samtools
MSIsensor	0.6	MIT License	https://github.com/ding-lab/msisensor
SSW	1.2.5	MIT License	https://github.com/mengyao/Complete-Striped-Smith-Waterman-Libr...

Setup an Analysis

Creating Analysis Parameters

1. Begin with selecting **Analysis** on the Navigation Bar to display the following Analysis Guide page.
2. User may skip parameter setting and select **Go to Start Analysis.**

The Custom parameter setting described in this Creating Analysis Parameters section is not required to start a run.

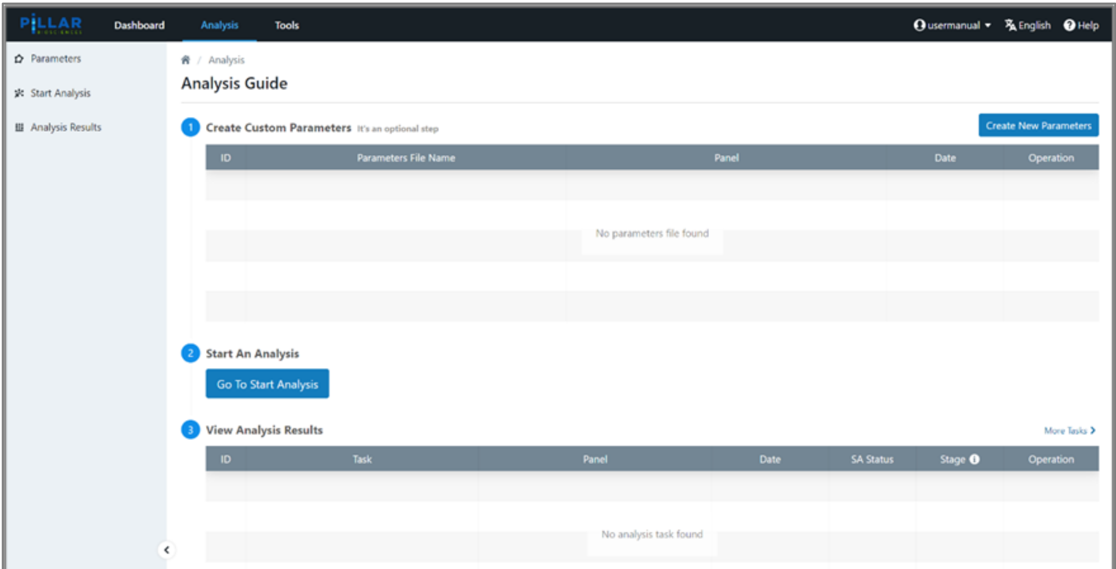


Figure 8 Analysis Guide page

3. Select **Create New Parameters** to display the following Analysis Parameters page. This enables user to customize analysis parameter.

- **Parameters Name:** Enter desired parameters file name. The user defined parameters will be saved with this name for future use.
- **Panel Selection:** Select desired panel using drop-down menu.

4. Select ① **Create New Parameters** and navigate across the tabs to customize settings for CORE_MODULE, Local Realignment, Paired End Assembly, Variant Call Reduce, Annotation/Filtering and CNV Processing.

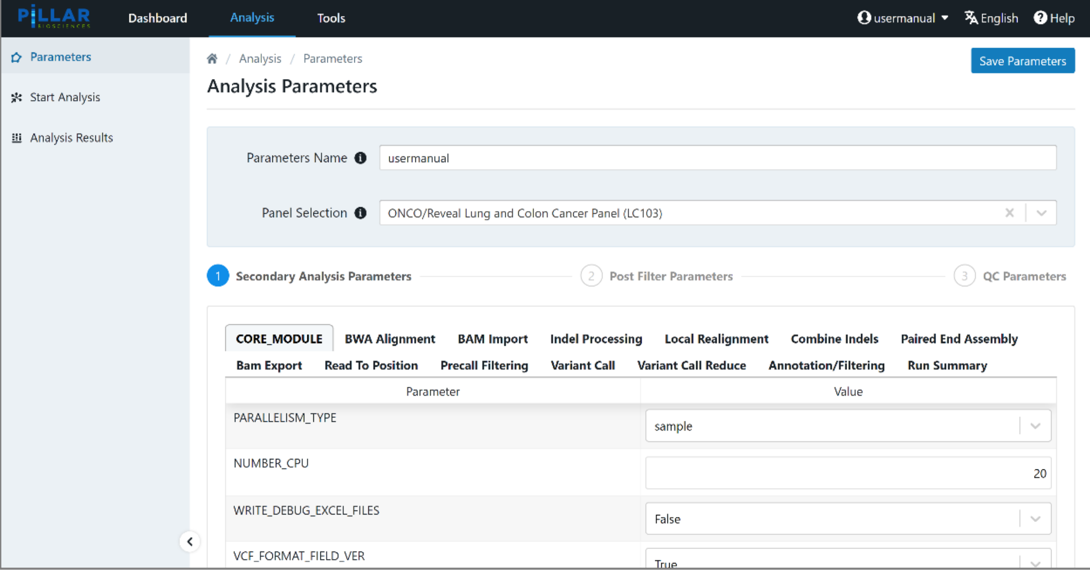


Figure 9 Analysis Parameters: ① Secondary Analysis Parameters page

5. Select ② **Post Filter Parameters** to customize settings for **Filtering**, **Grouping** and **Column Layout**.

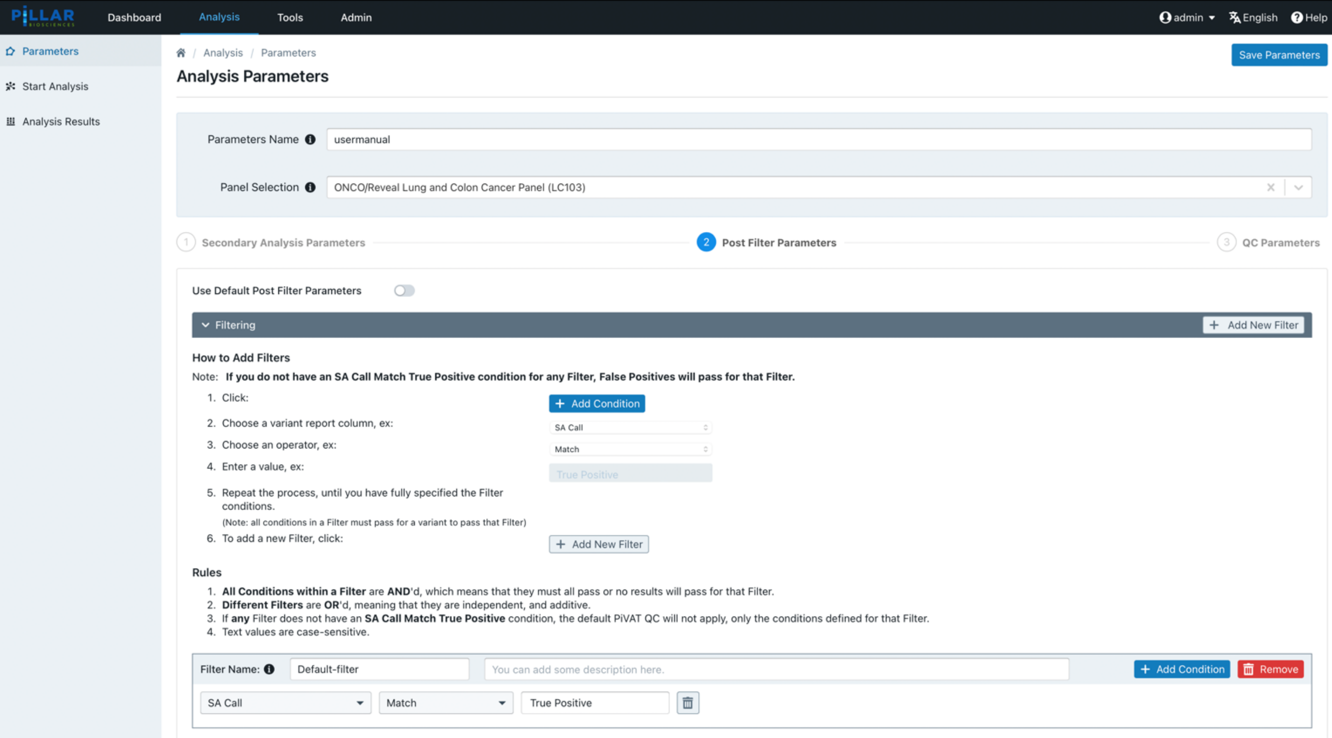


Figure 100 Analysis Parameters: ② Post Filter Parameters page

6. In ② **Post Filter Parameters,** use the CNV Threshold Grid to customize thresholds for filtering CNV calls. The CNV Threshold Grid will only appear if the selected panel is a CNV panel.

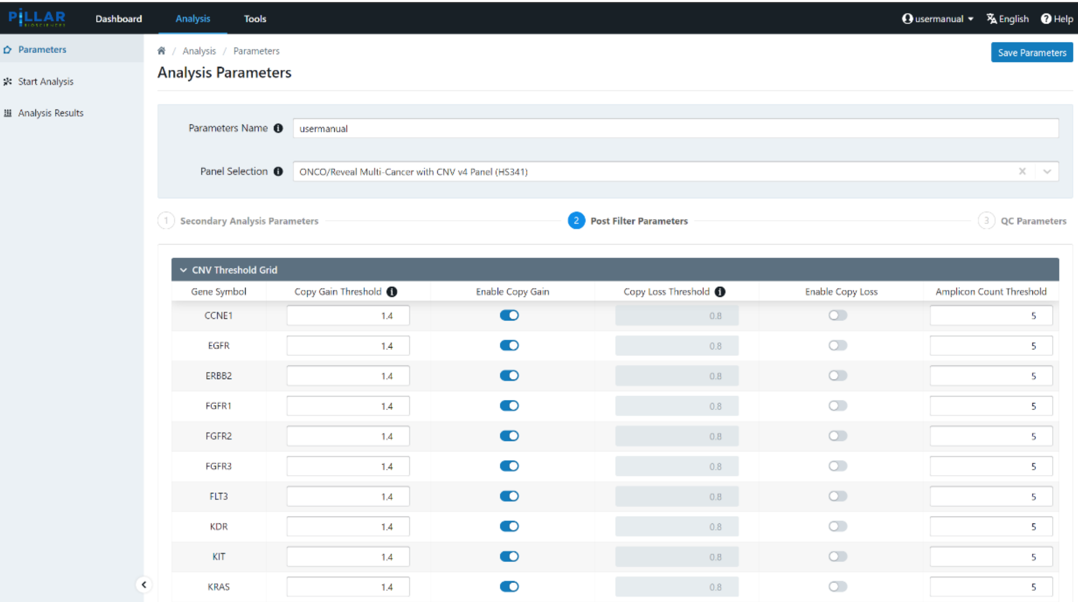


Figure 111 Analysis Parameters: ② Post Filter Parameters page CNV Threshold Grid

Note: When enabling Copy Loss for panels using the cfDNA CNV Caller (see Appendix C, currently only P-LBX-01): the **CNV_MAX_COPY_NUMBER** parameter will need to be adjusted to ensure that calls below the Copy Loss Threshold are reported. See Appendix C.

7. Select ③ **QC Parameters** to customize QC parameters for **Sample** and controls (**NTC**, **PosCtrl**, **NegCtrl**).

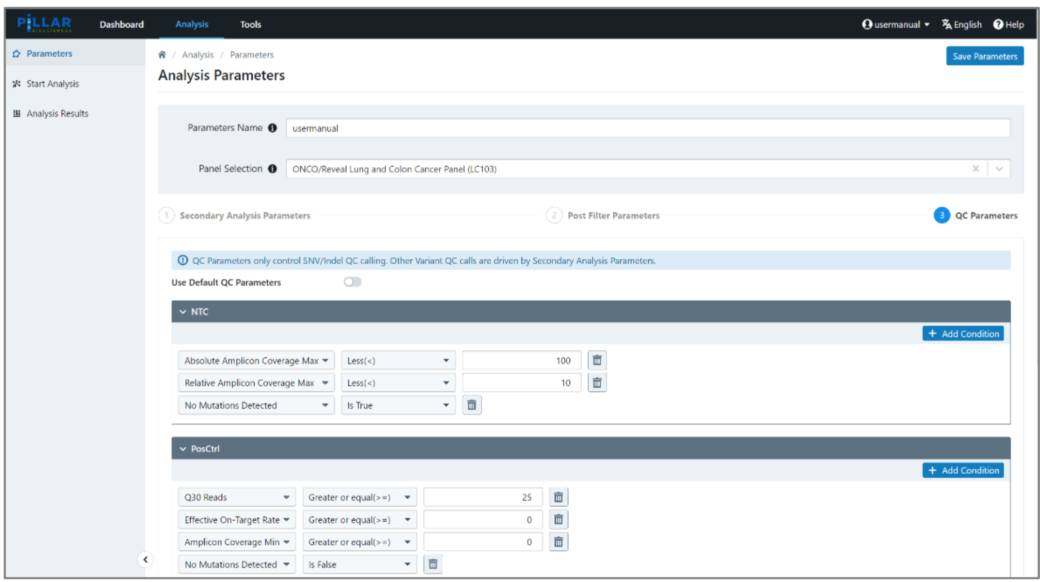


Figure 12 Analysis Parameters: ③ QC Parameters page

QC Parameters only control SNV/Indel QC calling. Other Variant QC calls are driven by Secondary Analysis Parameters.

Start Analysis

Please refer to the following appendices for caller-specific instructions.

- Appendix A: SNV/Indels Caller
- Appendix B: Microsatellite Instability
- Appendix C: Somatic CNV Caller
- Appendix D: Thalassemia CNV Caller
- Appendix E: SMA Thalassemia Caller

Appendix F: Fusion CallerAppendix E: SMA Caller

The SMA analysis is based on the double normalization method. The normalization baseline is calculated from negative reference samples. The SMA Caller calculates the copy number ratios of Exon-07 and Exon-08 amplicons on the SMN1 and SMN2 genes.

SMA Sample Setup

For each SMA analysis run, the user should provide 3-5 (minimum 2) in-run normal (negative) reference samples with similar sample condition and preparation process as the positive samples. If less than 2 negative reference samples are provided, the run will fail.

See CNV Sample Setup section in Appendix C: Somatic CNV Caller for instructions to define normal samples to be used as negative references.

SMA Analysis Setup

1. See CNV Analysis Setup section in Appendix C: Somatic CNV Caller for instructions to setup a SMA Thalassemia analysis.
2. The SMA_RESULTS excel file is output upon completion of the analysis.
3. Detailed SMA call information is in the "SMA Call Report" sheet of the SMA_RESULTS excel file.
4. Each sample's detailed SMA Report can be downloaded as a PDF file in the "Report" section of the results page.
5. The fully normalized copy number ratios can be found in "Fully Normalized" sheet in SMA_RESULTS excel file. Note that the copy number ratio in PiVAT is defined as the copy number ratio of a potentially positive sample to that of the negative reference samples (diploid with 2 copy number).

SMA QC parameters

See CNV QC parameters section in Appendix C: Somatic CNV Caller for a list of user adjustable QC parameters.

PiVAT Output: SMA_RESULTS file

Definition and/or description of result columns reported in SMA_RESULTS file sheets are provided below.

<table>
<tr><th>Sheet Tab</th><th>Column Name</th><th>Definition/Description</th></tr>
<tr><td rowspan="5">SMA Call Report</td><td>Sample ID</td><td>Unique Sample ID for each sample.</td></tr>
<tr><td>Gene-Exon</td><td>Name of the gene/exon pair, or if a control target, name of the control amplicon.</td></tr>
<tr><td>Location</td><td>Genomic coordinates of exon (hg19).</td></tr>
<tr><td>Copy Number</td><td>Copy number of the gene-exon or of the control amplicon</td></tr>
<tr><td>Copy Number Ratio</td><td>The copy number ratio of the gene-exon or control-amplicon to the copy number (2) of a negative normal sample.</td></tr>
<tr><td rowspan="2">SMA Run QC</td><td>QC Criteria</td><td>Indicate which run QC criteria that each row is reporting, including “Negative_Reference”, “Sample” and “Run_Status”.</td></tr>
<tr><td>QC Status</td><td>Whether the QC is passed for each run QC criteria. If run QC is passed, it is labeled as “Pass”. It run QC is failed, it is labeled as “Fail”.</td></tr>
<tr><td rowspan="3">Filtered SMA Samples</td><td>Sample ID</td><td>Unique Sample ID for each sample</td></tr>
<tr><td>Filter Reason</td><td>The reason that the sample is filtered out from the SMA Thalassemia analysis.</td></tr>
<tr><td>Sample Type</td><td>Indicate whether the reported sample is “Sample” or “Negative Reference”. This is pre-defined by the user before starting the SA analysis.</td></tr>
<tr><td>CNV Segment Coverages</td><td colspan="2">This sheet reports the raw segment coverages of each amplicon for all the samples initially added to the SMA analysis. The column headers are Amplicon Names and the row indices are Sample IDs.</td></tr>
<tr><td>Normalized Coverages</td><td colspan="2">This sheet reports the normalized copy number ratio of each amplicon for the samples that pass the sample QC. Note that the filtered samples are not reported in this sheet. The column headers are Amplicon Names and the row indices are Sample IDs.</td></tr>
</table>

SMA Calls Table

SMA Calls table contains the copy number ratios for the sample(s) selected in **Select Sample.**

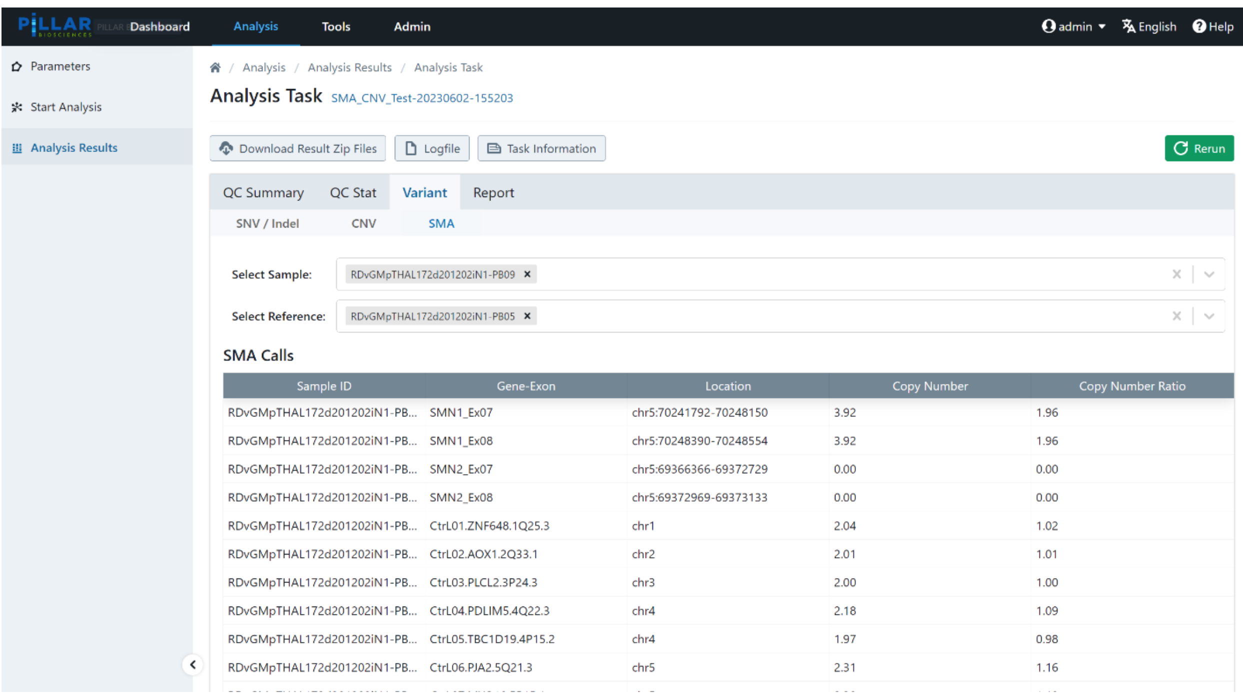


Figure 43 Analysis Results: SMA Calls table

Definition and/or description of result columns reported in the SMA Calls table are provided below.

Column Name	Definition/Description
Sample ID	Unique Sample ID for each sample.
Gene-Exon	Name of the gene/exon pair, or if a control target, name of the control amplicon
Location	Genomic coordinates of exon (hg19).
Copy Number	Copy number of the gene-exon or of the control amplicon
Copy Number Ratio	The ratio of the copy number of called Thalassemia CNV to the copy number (2) negative normal sample.

SMA Plot

A SMA plot for each sample will be located on the Variant > SMA tab below the SMA Calls table and SMA RUN QC table in Analysis Results. Each boxplot represents the copy number ratios of all CNV Normal Samples specified in the run, and the orange data points represent the copy number ratios of the potentially positive sample. The x-axis represents gene-exon labels, and the y-axis represents copy number ratio.

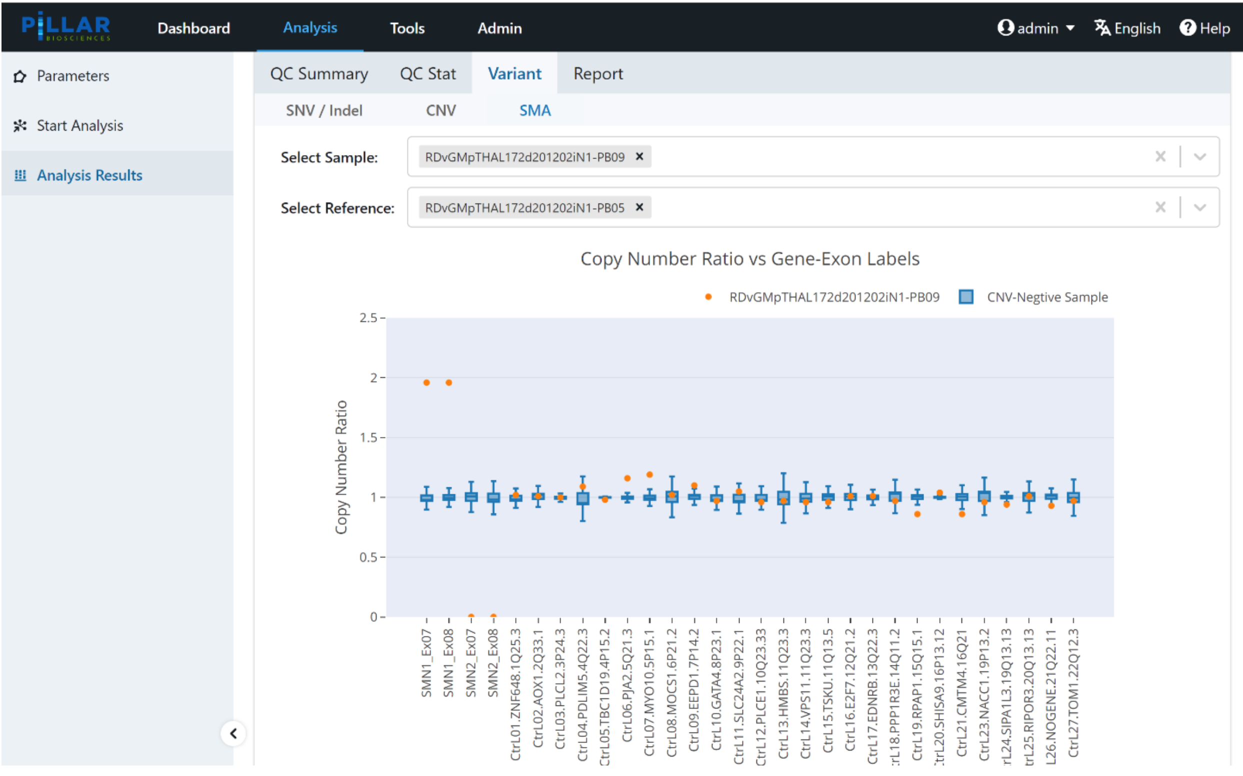


Figure 44 Analysis Results: SMA Sample Box Plot

To save the box plot graph, hover over the graph, and click the "**Download plot as a png**" icon as indicated below.

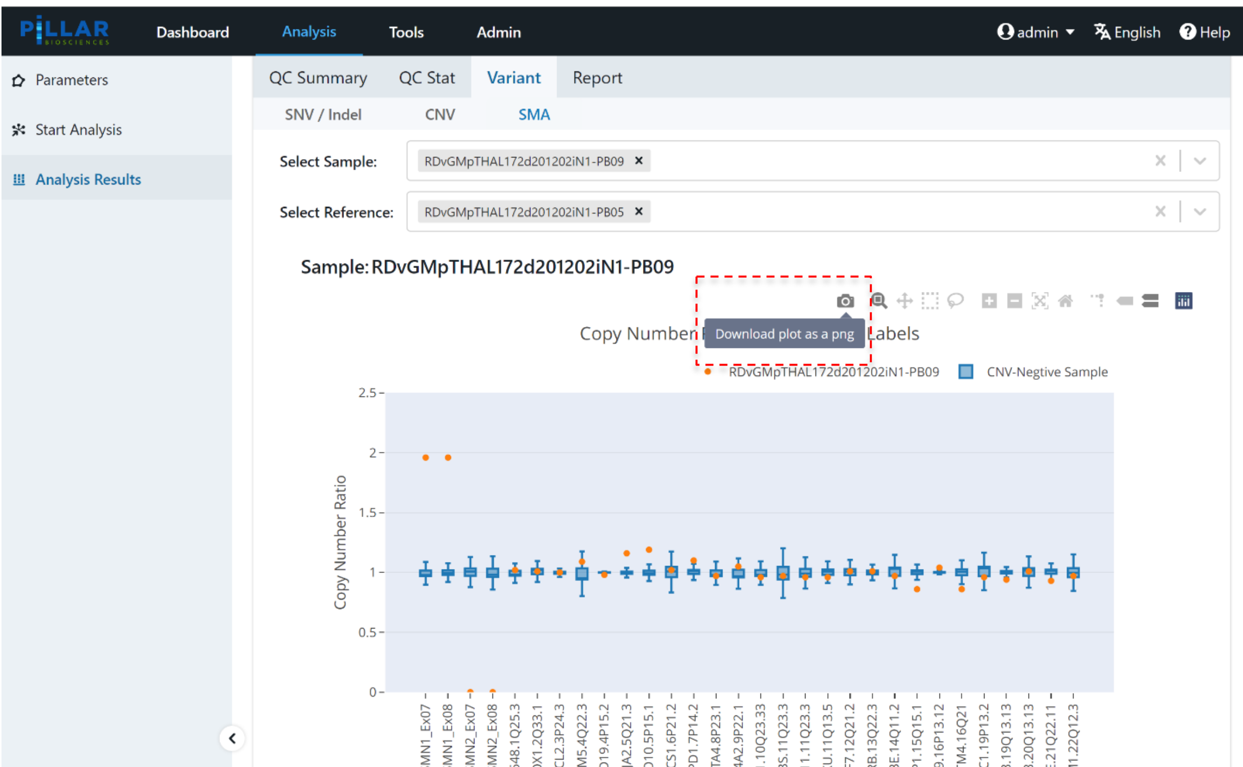


Figure 45 Analysis Results: SMA Sample Box Plot – Saving as PNG

- Appendix F: Fusion Caller

1. Select **Start Analysis** on the left menu bar to display the following Start Analysis: 1. Start From page.
   - **Select Panel:** Select desired panel from the drop-down menu.
   - **Parameters File**: Parameters file selection is optional. Refer to Creating Analysis Parameters section for instructions to create custom parameters file. Default parameters will be applied if parameters file is NOT selected.
   - **Select Samples**: Select desired FASTQ OR BAM format files. Refer to Data Management section for instructions to import data.

⚠ BAI (BAM Index) files may only be used as PiVAT input if accompanied by their respective BAM files. Using only BAI files without their respective BAM files will result in a run failure.

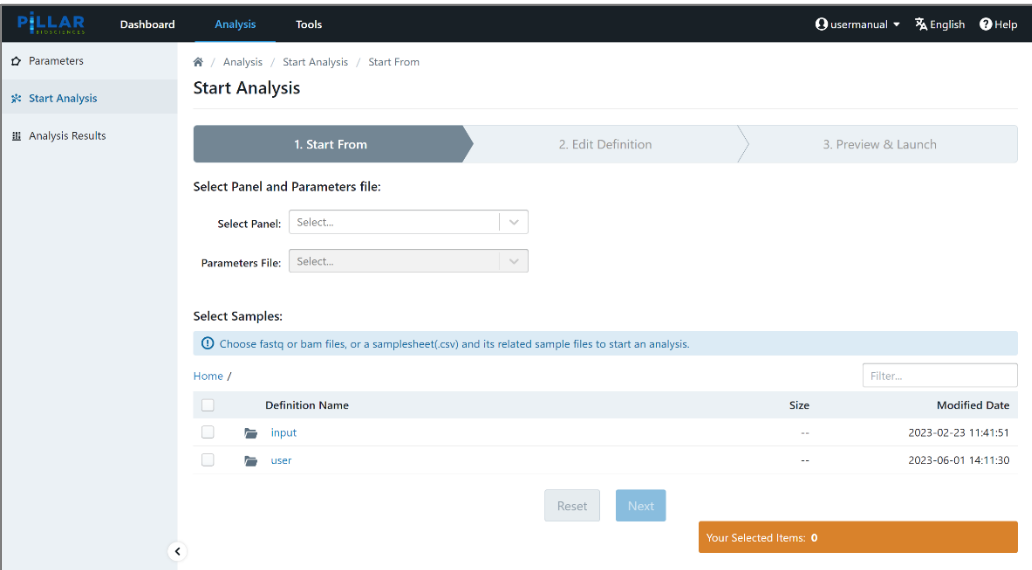


Figure 13 Start Analysis: 1. Start From page

2. Select **Next** to display the following window: 2. Edit Definition page.
   - **Analysis Name:** Enter desired name for the analysis to uniquely identify the run.
   - **Parameters File**: Parameters file selection is optional. Refer to Creating Analysis Parameters section for instructions to create custom parameters file. Default parameters will be applied if parameters file is NOT selected.
3. Users may edit sample information such as **Sample Name**, **QC Type** as well as edit or skip **Sample Files**.
4. Users may also select samples using check boxes to define **Tumor-Normal Paired** and **CNV Normal Samples**.

Tumor-Normal Paired definition is required for performing MSI analysis, refer to Appendix B: Microsatellite Instability (MSI) Caller for details.

CNV Normal Samples definition is recommended for CNV analysis, refer to Appendix C: Somatic CNV Caller for details.

CNV Normal Samples definition is required for Thalassemia CNV analysis, refer to Appendix D: Thalassemia CNV Caller for details.

P-LBX-01 requires at least 1 CNV Normal to be specified (using Define CNV Normal Samples button as seen in the image below). If sample tumor content is known, it should be specified in the **Tumor Content %** column (as a percentage) for improved CNV calling performance.

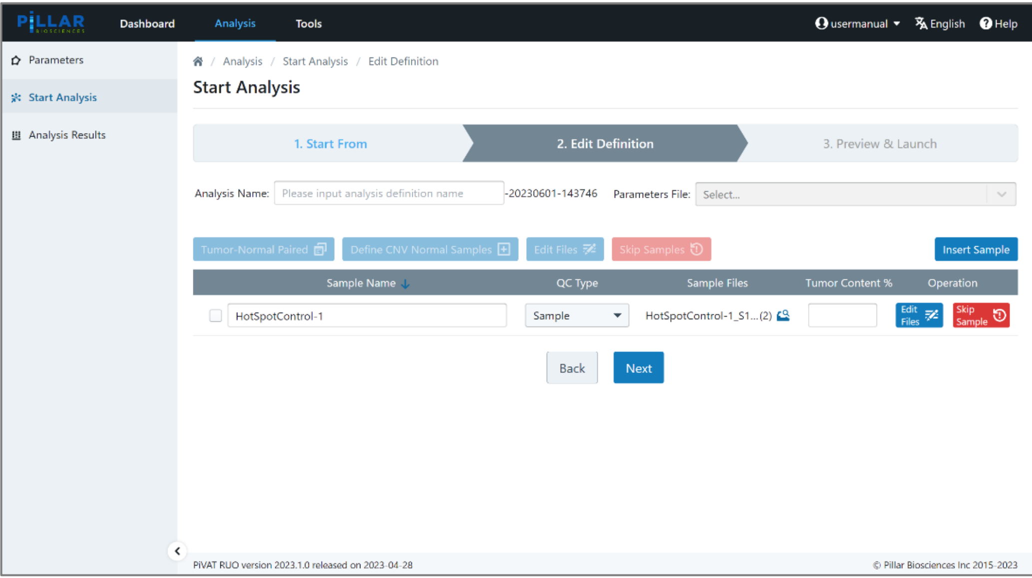


Figure 14 Start Analysis: 2. Edit Definition page

5. Select Next to display the following window: 3. Preview & Launch page. Once user confirms the analysis setup, select **Launch Analysis**.
6. A pop-up message will appear. User may select:
   - **Start Another Analysis** to redirect to **Start Analysis** page, or
   - **Go to Analysis Results** to redirect to **Analysis Results** page.

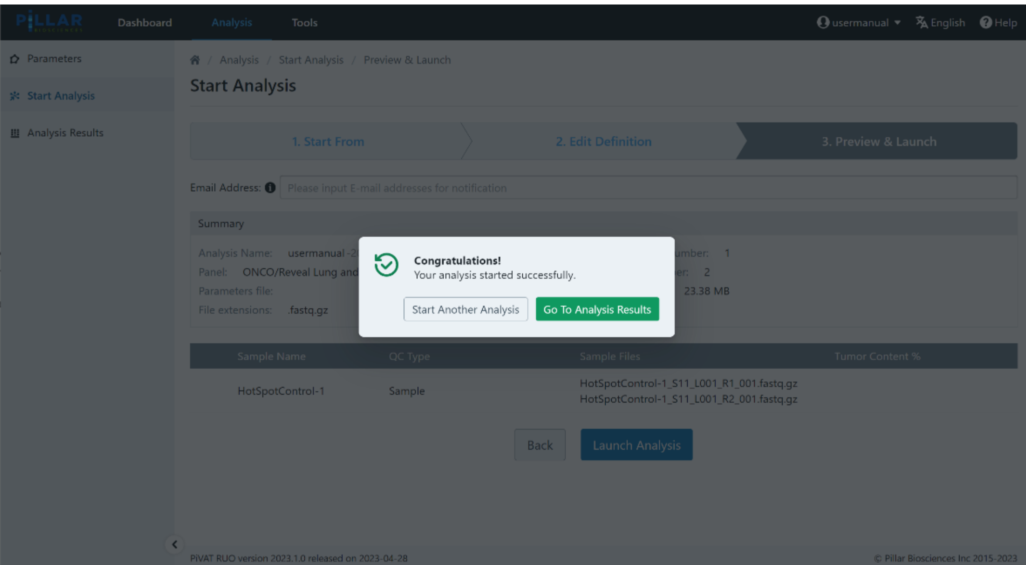


Figure 15 Start Analysis: 3. Preview & Launch page

Monitoring the Task Status

1. The PiVAT Dashboard will display summary of analysis tasks, data storage and **Recent Task Activity** of the user currently logged in.
2. To view the complete list of order task, select **More Tasks >**.

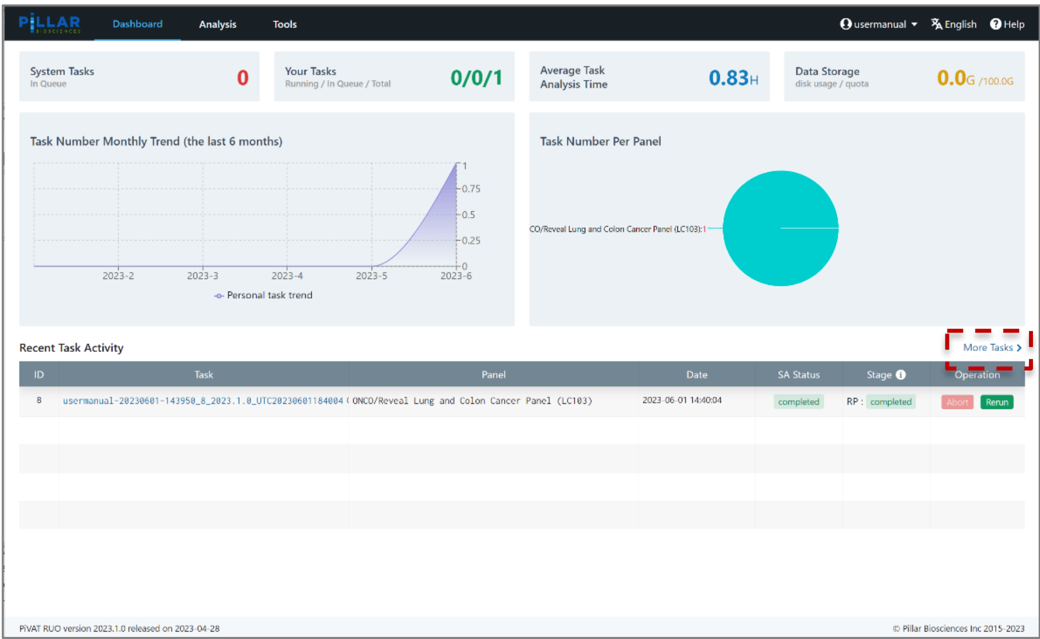


Figure 16 Dashboard page

3. The following information is provided for each task row:

- **ID:** Unique task ID number for current database.
- **Task:** Analysis name entered by user during analysis setup.
- **Panel:** Panel selected for the analysis.
- **Date:** Date when the analysis task was created.
- **Status**: Analysis task status
  - ✓ **queued**: There are other task(s) running. This task is queued until preceding tasks are finished.
  - ✓ **running**: This analysis is currently running.
  - ✓ **completed**: The analysis has completed successfully.
  - ✓ **failed**: An error occurred, and the analysis did not complete.
  - ✓ **aborted**: This task was aborted by a user.
- Stage: Where in the Analysis the task currently is. Only displayed if in the **running** or **failed** state.
  - ✓ **SA:** Secondary Analysis Pipeline
  - ✓ **FA:** Functional Annotation
  - ✓ **PF:** Post Filter
  - ✓ **QC:** Quality Control
  - ✓ **RP:** Report Generation
- **Operation**: User may select **Abort** to terminate an analysis task in running state or **Rerun** to repeat an analysis as-is or with edits to analysis setup.

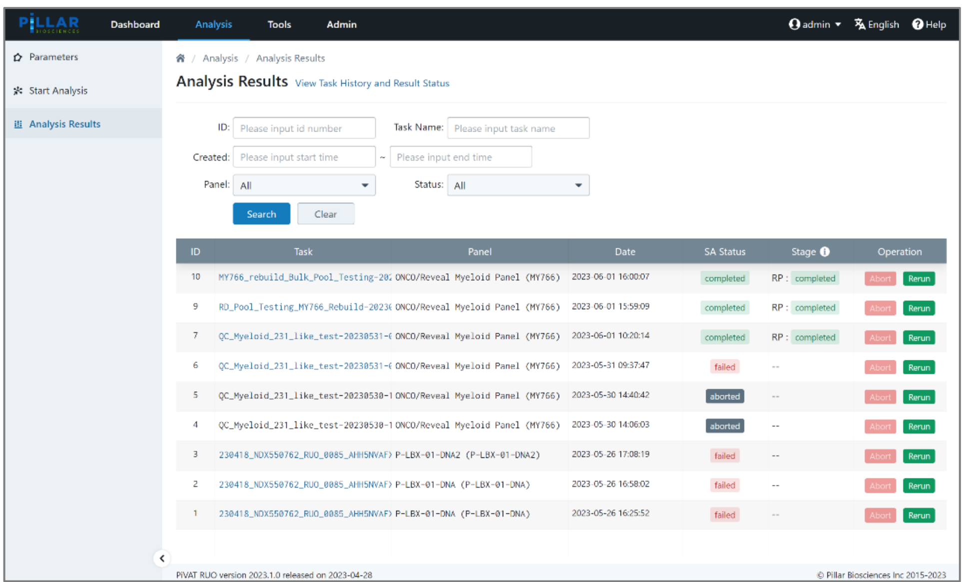


Figure 17 Analysis Results: Task History page

View Summary of Task Details

1. Hover the mouse over the task name to view a pop-up summary of analysis.

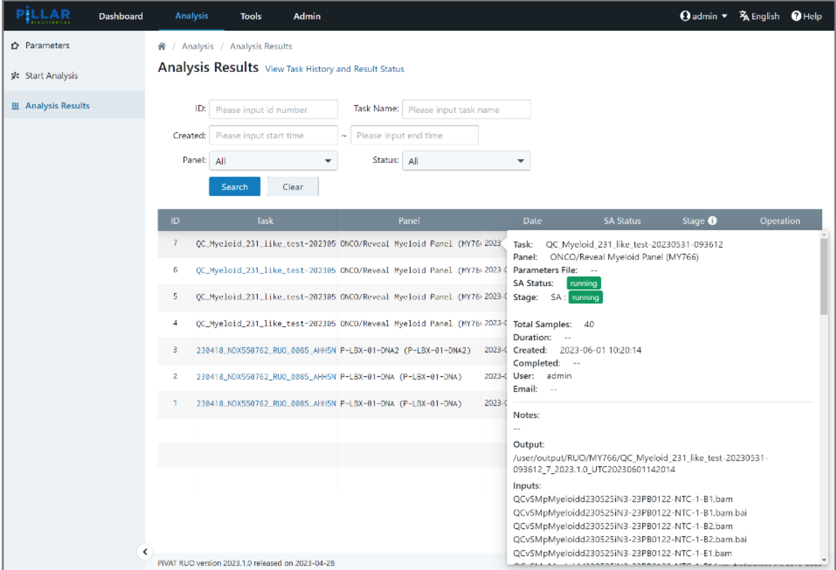


Figure 18

2. The summary information includes:

- Task name
- Panel
- Parameters file name
- Task status
- Stage
- Total number of Samples
- Duration of the run
- Created and Completed date/time stamps
- Note that the time between the Created and Completed time stamps may be longer than the Duration if the task was queued before starting
- The user who ran the task
- Email address if configured
- The output folder location
- Input files
- Arguments

View Completed Analysis Task

1. Select task hyperlink from list of completed tasks to display the Analysis Task page. Users may download analysis results, view log file and task information or rerun an analysis task.

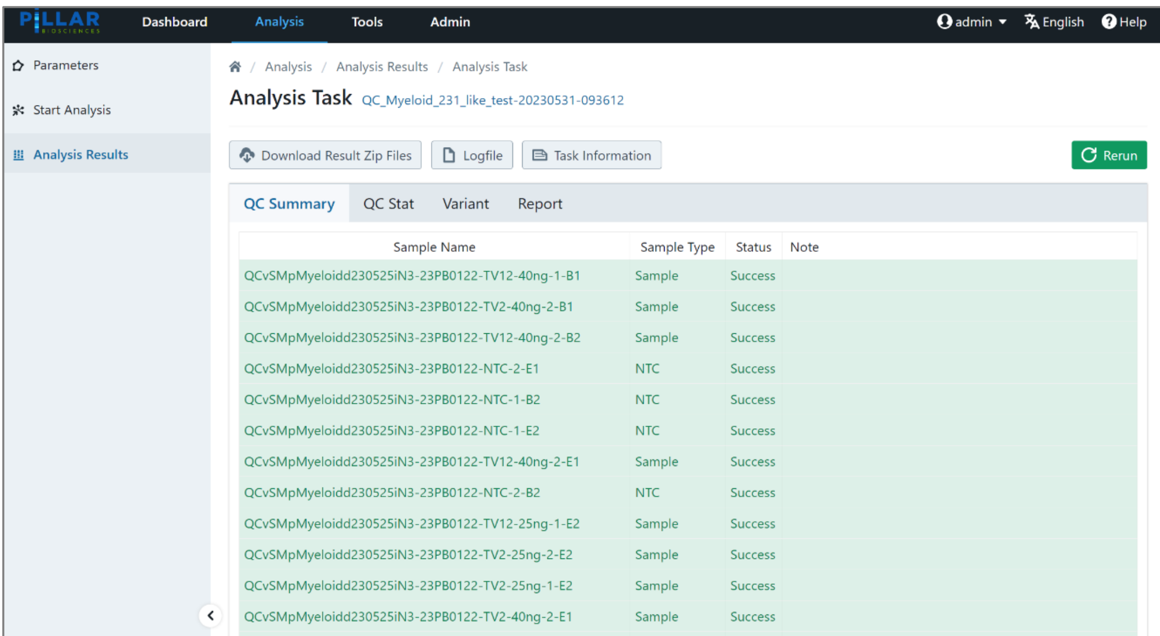


Figure 19 Analysis Task page

2. Click the **Download Result Files** button to download a zip formatted file with the files produced by the analysis run.

File download is unavailable if the task is in the **queued** or **deleted** status.

Users may delete the zip file from this screen. Please verify that analysis data has been downloaded and stored in accordance with your organization's IT policies *before* deleting data from the PiVAT system.

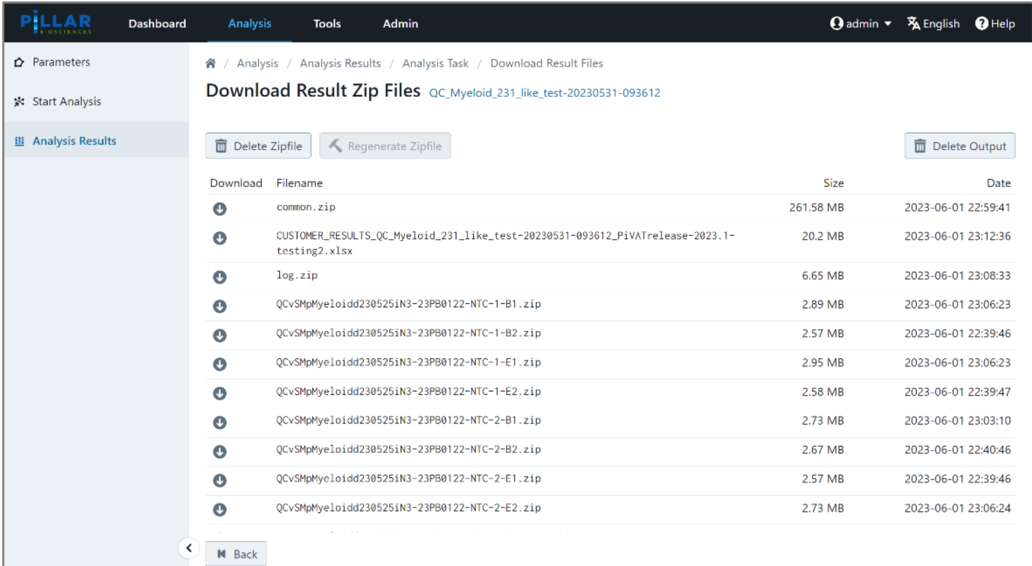


Figure 20 Analysis Task: Download Result Files page

3. A deleted zipfile may be recovered by selecting the **Regenerate Zipfile** button. This selection is only available if the files are still present on the system. If the output zipfiles have been deleted, the regular output files may still be available via your network drive or through SFTP. Refer to the Help page to learn more about SFTP.
4. Click the **Back** button or 🏠 Analysis / Analysis Results / **Analysis Task** to return to Analysis Task page.
5. Click the **Logfile** button from Task Analysis page to display information for every stage of this task. The information is not available when the task is in the running, queued or deleted status.
6. Click the **Back** button or 🏠 Analysis / Analysis Results / **Analysis Task** to return to Analysis Task page.

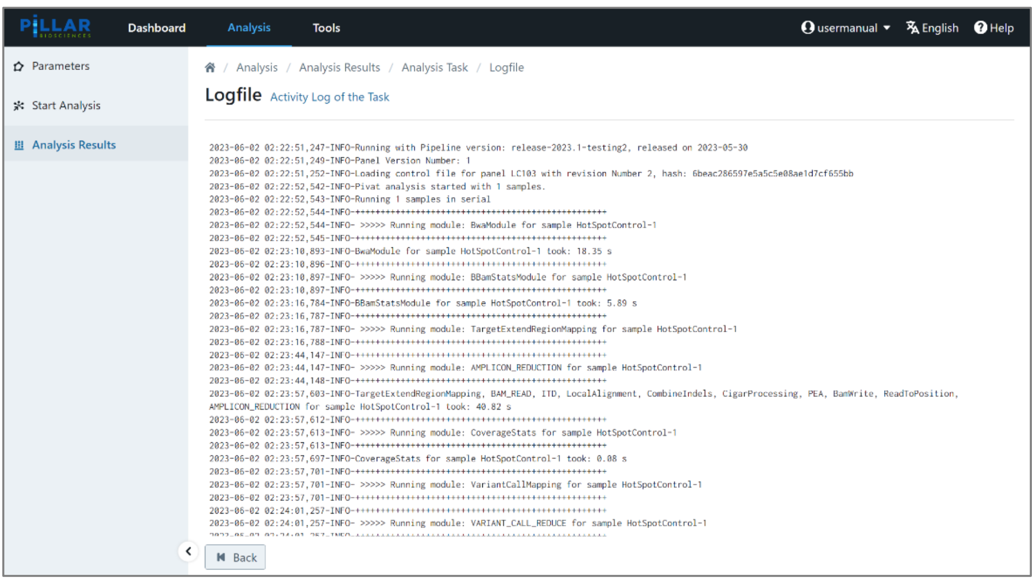


Figure 21 Analysis Task: Logfile page

7. Click the **Task Information** button to display the summary of analysis, this is the same information you get by hovering mouse over the task name on Analysis Result page.

8. Click the **Back** button or 🏠 Analysis / Analysis Results / **Analysis Task** to return to Analysis Task page.

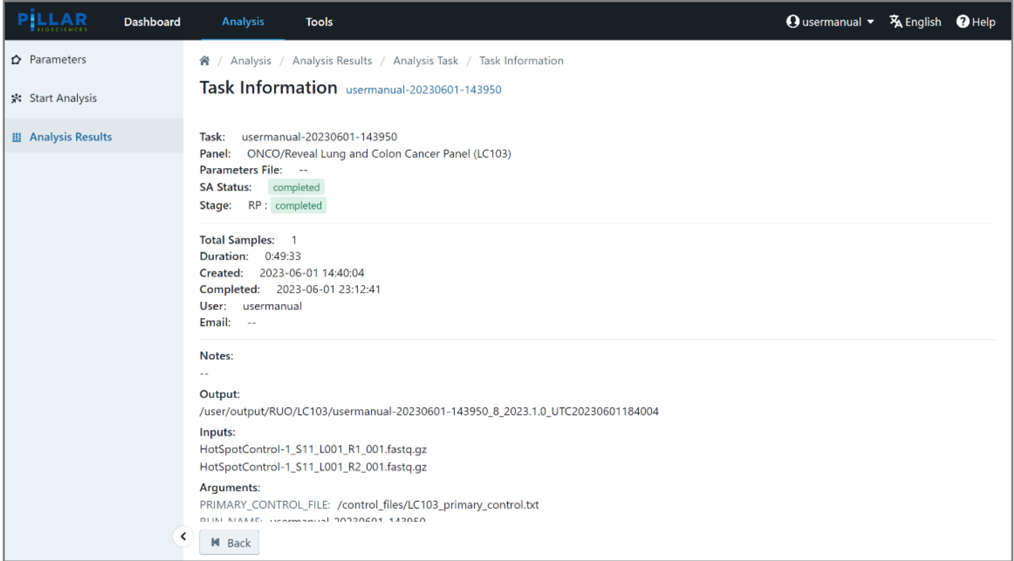


Figure 22 Analysis Task: Task Information page

9. Click the **Rerun** button for the option to repeat the analysis as-is or with edits to analysis setup.

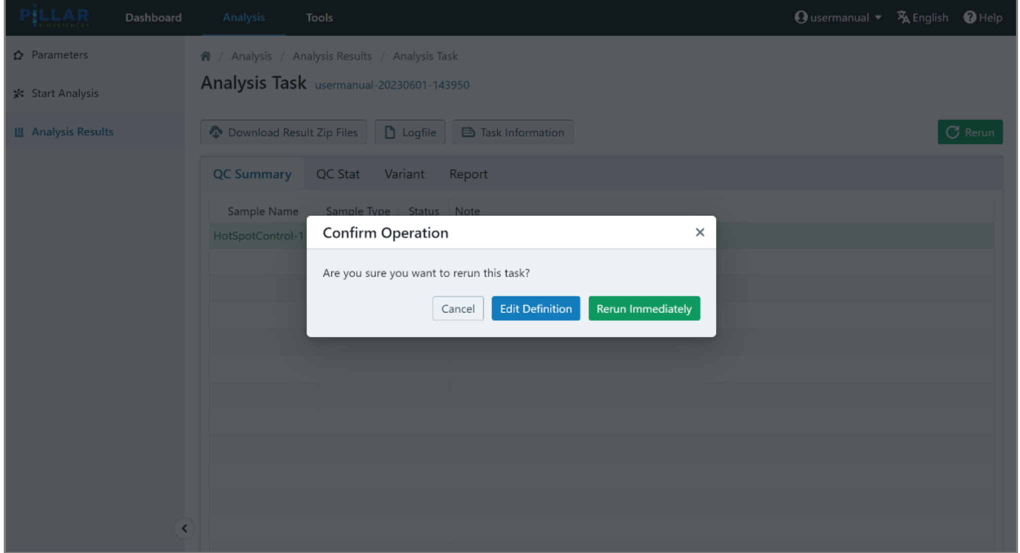
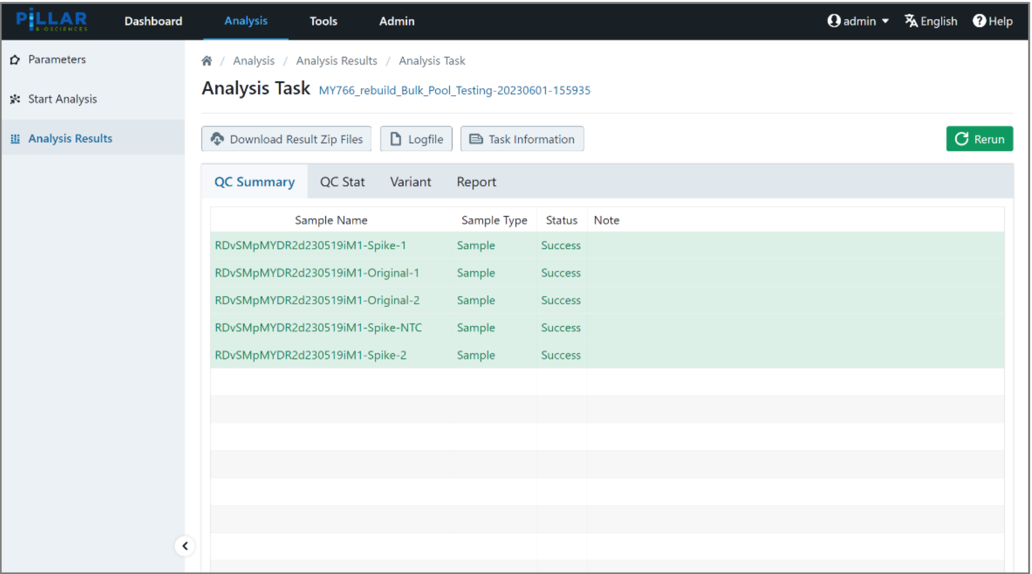


Figure 23 Analysis Task: Rerun confirmation message

View Analysis Results

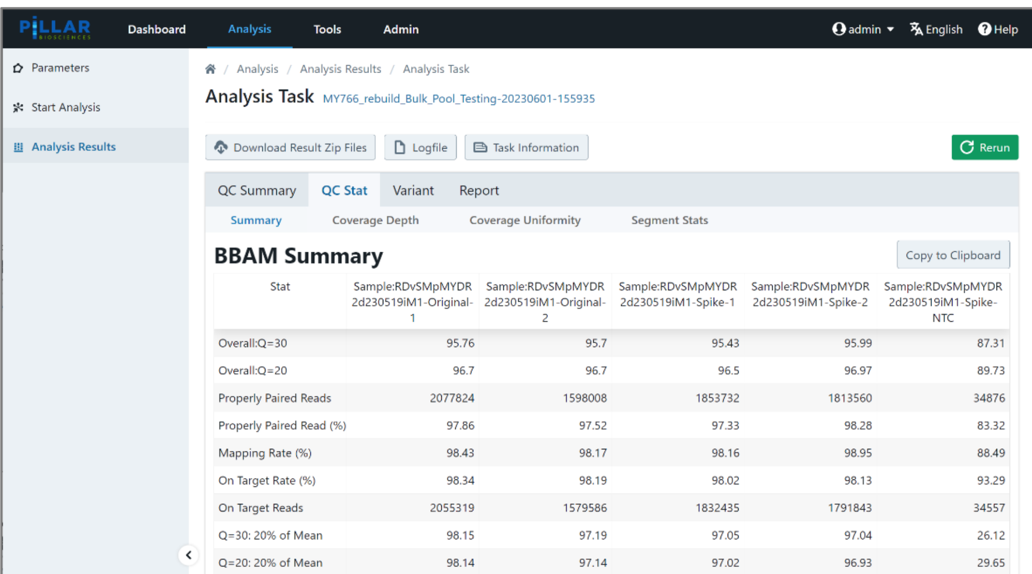
1. Users may navigate across the tabs to view QC Summary, QC Stat, Variant and Report.
2. Click the **QC Summary** tab to display summary of Sample Type and Status for each sample.



Sample Name	Sample Type	Status	Note
RDvSMpMYDR2d230519iM1-Spike-1	Sample	Success	
RDvSMpMYDR2d230519iM1-Original-1	Sample	Success	
RDvSMpMYDR2d230519iM1-Original-2	Sample	Success	
RDvSMpMYDR2d230519iM1-Spike-NTC	Sample	Success	
RDvSMpMYDR2d230519iM1-Spike-2	Sample	Success	

Figure 24 Analysis Task: QC Summary tab

3. Click the **QC Stat** tab to display and navigate sub-tabs to view Summary, Coverage Depth, Coverage Uniformity, and Segment Stats.



Stat	Sample:RDvSMpMYDR 2d230519iM1-Original-1	Sample:RDvSMpMYDR 2d230519iM1-Original-2	Sample:RDvSMpMYDR 2d230519iM1-Spike-1	Sample:RDvSMpMYDR 2d230519iM1-Spike-2	Sample:RDvSMpMYDR 2d230519iM1-Spike-NTC
Overall:Q=30	95.76	95.7	95.43	95.99	87.31
Overall:Q=20	96.7	96.7	96.5	96.97	89.73
Properly Paired Reads	2077824	1598008	1853732	1813560	34876
Properly Paired Read (%)	97.86	97.52	97.33	98.28	83.32
Mapping Rate (%)	98.43	98.17	98.16	98.95	88.49
On Target Rate (%)	98.34	98.19	98.02	98.13	93.29
On Target Reads	2055319	1579586	1832435	1791843	34557
Q=30: 20% of Mean	98.15	97.19	97.05	97.04	26.12
Q=20: 20% of Mean	98.14	97.14	97.02	96.93	29.65

Figure 25 Analysis Task: QC Stat tab

4. Click the **Variant** tab to display and navigate sub-tabs to view variant results. Variant results grouping will be displayed according to the Parameters File applied.

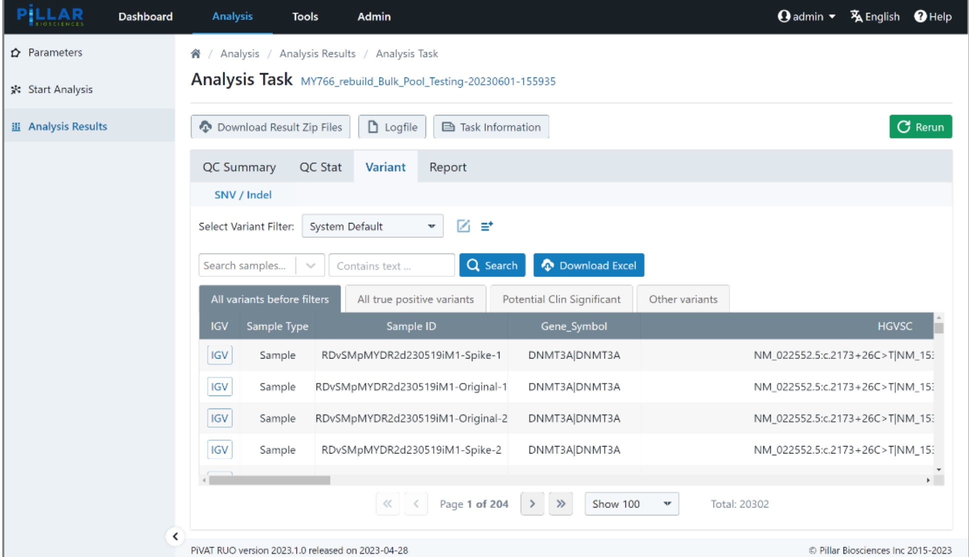


Figure 26 Analysis Task: Variant tab

5. Click the **Report** tab to view list of samples and option to download all or selected reports.

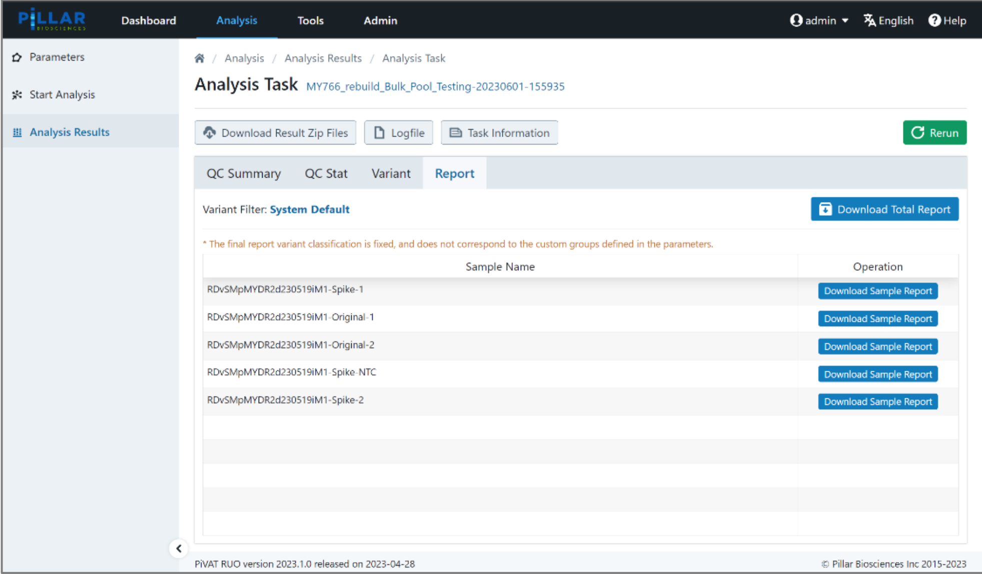


Figure 27 Analysis Task: Report tab

User password

1. A user can change their own password by selecting the **Profile** option from the drop-down menu for the username. The following page is displayed:

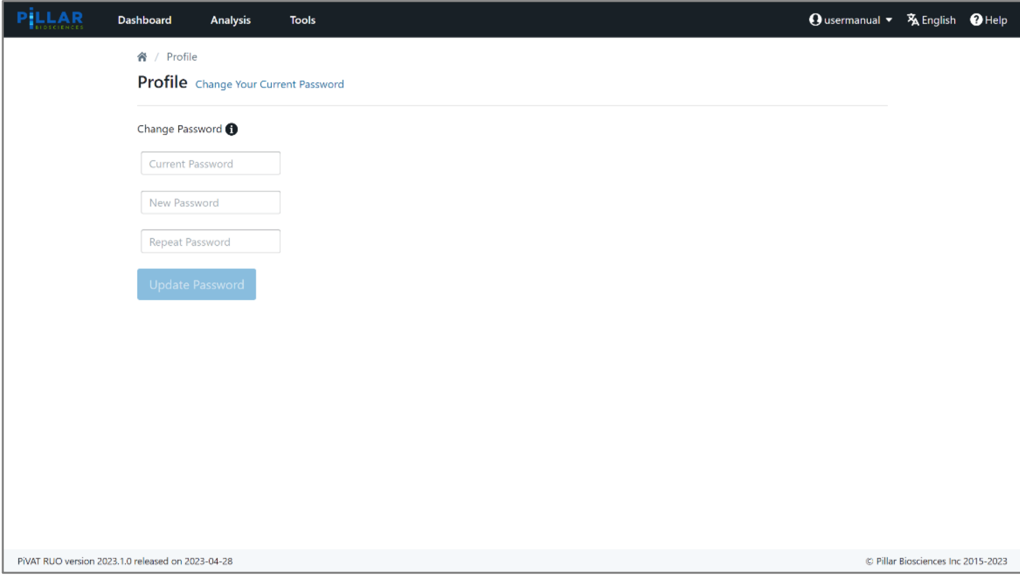


Figure 28 Profile: Change Your Current Password page

2. Create new password and then click **Update Password** to confirm.

3. To exit without changing your password, click 🏠 or any option from the Navigation Bar.

Record the new password in accordance with your organization's IT policies.

Data Management

1. Begin by clicking **Tools** on the Navigation Bar and **Data Management** on the left menu bar to display the following Data Management page.
2. The Data Management page is used to upload input data (FASTQ) to the PiVAT system for analysis.
3. Storage data is displayed for verification of sufficient free storage before attempting to upload data.

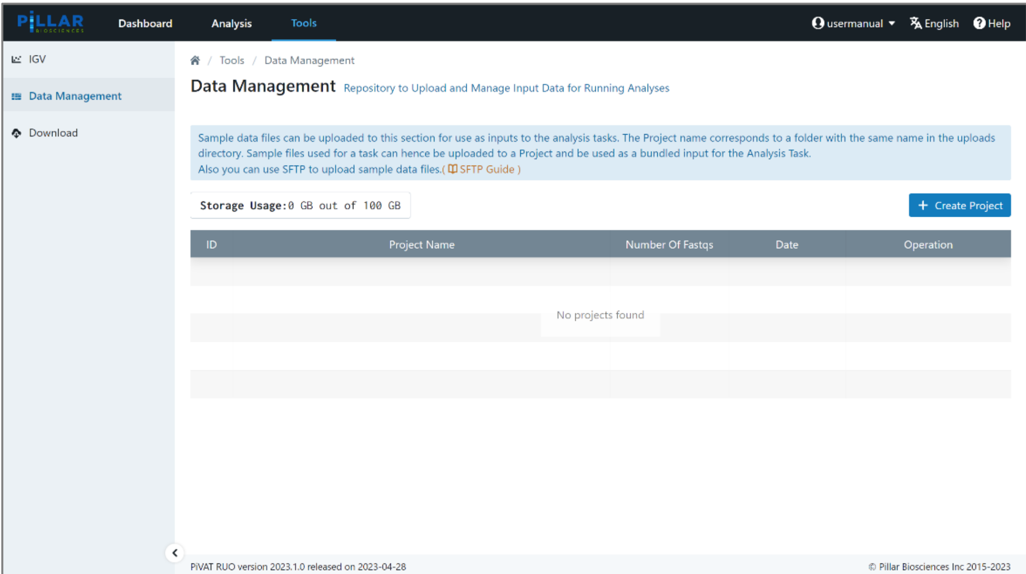


Figure 29 Data Management page

4. Input data can be uploaded to a Project listed on the page. To create a new project, click **+ Create Project**.
5. All created projects are listed on this page. Click the Project Name you wish to upload data to and the following page will be displayed:

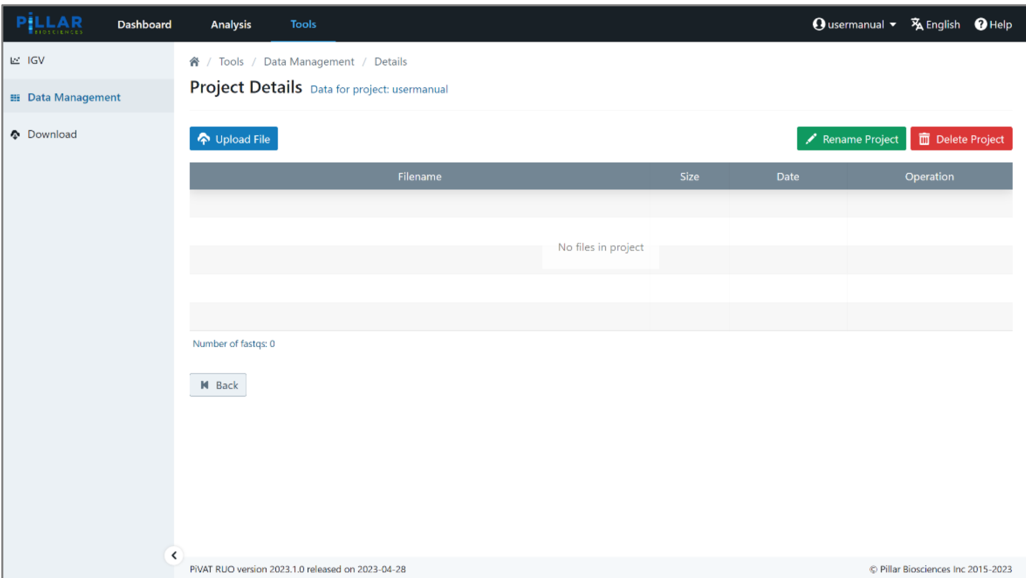


Figure 30 Project Details page

6. Click the **Upload File** button to display the Upload Input Files page. From here users may drag & drop files or select the files for upload. Once your files are displayed on the page, select **Start Upload**.

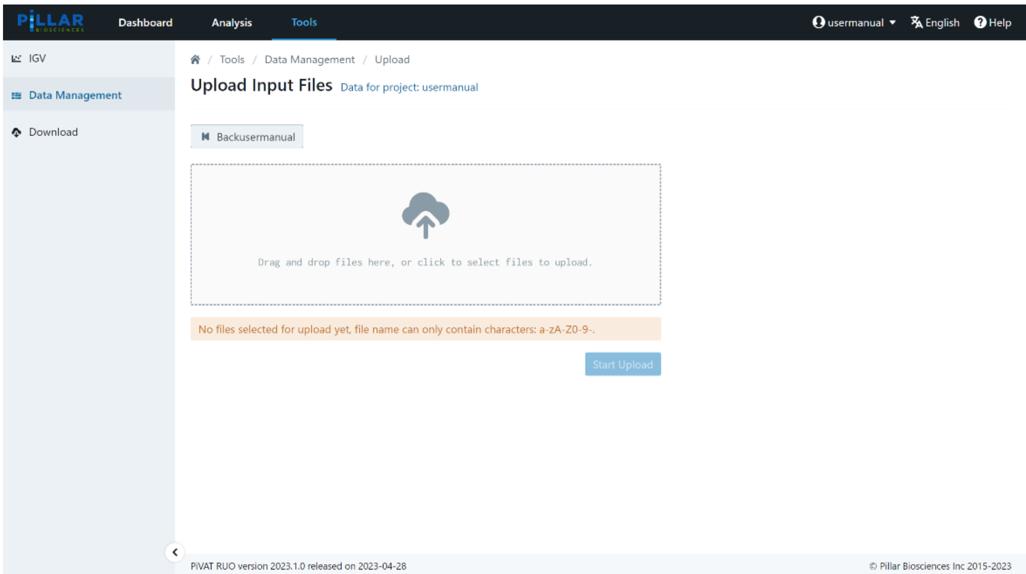


Figure 31 Upload Input Files page

7. Users may also select **Rename** or **Delete** the project from the Project Details page

Take caution when deleting a project, as it will result in all data within the project to be deleted.

Selection of directories for upload is only supported by the Drag and Drop option.

SFTP Download

To download files using SFTP (Secure File Transfer Protocol), your installation of PiVAT must have the SFTP feature enabled by the administrator. Please contact your administrator to confirm if this feature is available. To use the SFTP feature, SFTP client software is required such as FileZilla or Cyberduck. Use the following settings.

Address: sftp://*[IP address of your PiVAT system]*
Port: 2225
Username: Your PiVAT username
Password: Your PiVAT password

When uploading is interrupted, the file number and total size may be incorrect, please logout & login to the SFTP client again so the system can refresh. Deletion of corrupted files will also require you to logout and login again.

Some SFTP clients, FileZilla for example, may run into an infinite loop of retrying an upload when a file exceeds the size limitation. Please terminate it manually.

Troubleshooting

If the system requires a restart, the user should check that all running and queued analysis tasks have completed before restarting the system.

If a task fails due to insufficient disk space, an administrator user can free up space on the system. There must be at least 100 GB of free space on the system for a task to start.

To avoid performance issues, do not run other applications on the workstation while PiVAT is running.

If the PiVAT application will not load, contact Pillar Biosciences at (800) 514-9307 or support@pillarbiosci.com.

If PiVAT is nonresponsive, it is possible to restart PiVAT using the following command:

- Open a terminal window
- cd /pillar/docker_files
- sudo docker-compose down
  - Superuser password required
- sudo docker ps
  - Verify that no docker containers are active
- sudo docker-compose up -d
- sudo docker ps

- Verify that the required containers are now running.

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Chrome is a registered trademark of Google.
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Natick, MA 01760

(800) 514-9307
support@pillarbiosci.com
https://pillarbiosci.com

Appendix A: SNV/Indels Caller

The SNV/Indels calling analysis reports all SNV and indel variant calls that have passed PiVAT's filtering criteria based on parameters set. The output will display true positive SNV and indel variants and annotations.

SNV/Indels Analysis Parameters Setup

Please refer to Creating Analysis Parameters section on page 11 for instructions on how to create and save analysis parameters.

1. Navigate to ① **Secondary Analysis Parameters** page to access the following list of adjustable parameters and default values in [ ] by tab.

Tab	QC Parameters [default]
CORE_MODULE	**VCF_FORMAT_FIELD_VER [True]** This parameter collapses all reports of a mutation across samples to a single entry in VCF files, using the FORMAT field. Default is set to True.
Local Realignment	**SOFT_CLIP_COUNT (count) [10]** The number of same soft clips threshold to keep the data. Default is set to 10.
Paired End Assembly	**PAIRED_READ_ONLY [False]** This parameter is to set if only paired end reads will be accepted for calling. Default is set to False.
Variant Call Reduce	**VCR_FILTER_QUALITY (q-value)** Minimum variant quality acceptance. Default value may change depending on the chosen panel for the analysis.
Annotation/Filtering	**VCR_FEATURE_FILTER [True]** Filters variants by the Feature column with NM identifiers. Default is set to True.
	VCR_FILTER_QUALITY (q-value) Minimum variant quality acceptance. Default value may change depending on the chosen panel for the analysis.
	VCR_FILTER_FREQUENCY (pct) Minimum variant frequency. Default value may change depending on the chosen panel for the analysis.
	VCR_FILTER_REPEAT_FREQUENCY (pct) Minimum variant frequency for repeat regions. Default value may change depending on the chosen panel for the analysis.
	VCR_FILTER_COVERAGE (count) [50] Minimum total coverage. Default is set to 50.

Tab	QC Parameters [default]
	VCR_FILTER_MIN_RATIO [-2] Minimum variant read direction ratio. -2 implies variant is on the reverse read while reference on the forward read. 0 means no preference for forward or reverse read of variant with respective to the reference. Default is set to -2.
	VCR_FILTER_MAX_RATIO [2] Maximum variant read direction ratio. Setting to 2 implies variant is on the reverse read while reference on the forward read. Setting to 0 means no preference for forward or reverse read of variant with respective to the reference. Default is set to 2.
	VCR_FILTER_VARIANT_COVERAGE (count) Minimum variant coverage. (Total Coverage * Variant Read Frequency)
	VCR_REPEAT_REGION_QUALITY_FILTER (q-value) [30] Minimum variant quality within a repeat region. Default is set to 30.
	SM_OVERLAP_THRESHOLD [5] The threshold parameter is used for merging variants from overlapping amplicons. If a single variant has a frequency above this value and all others are below, that variant is taken alone, otherwise all variants are merged together. Default value is set to 5.
	FLANKING_DISTANCE [5000] The distance of upstream and downstream between a variant and a transcript for which VEP will assign the upstream_gene_variant or downstream_gene_variant consequences. Default is set to 5000.
	CUSTOM_ANNOTATION_FLAG [False] This parameter is to set whether custom VEP annotations should be run. Default is False.

PiVAT Output: CUSTOMER_RESULTS file

Definition and/or description of result columns reported in CUSTOMER-RESULTS file sheet tabs are provided below.

Sheet Tab	Column Name	Definition/Description
Variant Report	Sample_ID	User defined library ID, derived from FASTQ/BAM file name
	SampleType	Type of sample used for analysis. i.e., Sample, NTC, PosCtrl, or NegCtrl
	Chromosome	Chromosome number in human genome where variant occurs
	Position	Base position on given chromosome where variant occurs (hg19)
	REF	Sequence found in human reference genome (hg19)
	ALT	Variant sequence found in sample
	Location	Genomic coordinates of variant (hg19)
	Variant_Type	SNV = single nucleotide variant; Deletion = one or more base deletion; Insertion=one or more base insertion; Delins = "deletion-insertion", variant characterized by both a deletion of one or more bases AND an insertion of one of more bases on the same allele. i.e., AGACTA ---> ATTCTA resulting from deletion of GA and insertion of TT or AGACTA ---> ATCTA resulting from deletion of GA and insertion of T
	VARIANT_CLASS	Sequence Ontology (SO) terms to identify variant type
	Variant_Length	Number of affected bases
	Variant_Net_Length	The net length between the difference of a variant between the length of the reference sequence and the alternate sequence. Variant_Net_Length = (length of alternate sequence – length of Reference sequence)
	Amplicon_ID	Amplicon in assay in which variant occurs.
	Variant_Read_Frequency_(%)	Occurrence of variant in sequencing data, as a percentage of total sequenced reads in the given segment. (Variant_Coverage / Total_Coverage)*100
	Variant_Coverage	Number of reads that contain variant in segment.
	Total_Coverage	Total number of reads in segment.
	Variant_Quality	Measure of variant call quality. Score is from 0 (low quality) to 40 (highest quality)

Sheet Tab	Column Name	Definition/Description
	Variant_Read_Direction_Ratio	Measure of strand bias in variant calling. Value of 0 indicates variant was found on both the positive strand and negative strand at equal rates (ideal). Values greater than zero or less than zero indicate percentage of bias towards the negative strand (negative values) or positive strand (positive values)
	Zygosity	Germline mutation panels only. HETEROZYGOUS = one out of two alleles contain variant; HOMOZYGOUS = both alleles contain variant
	Consequence	Type of mutation. Examples: frameshift_variant = variant produces an mRNA transcript that is out of the normal reading frame. synonymous_variant = variant does not affect amino acid sequence of final protein due to redundancy of codons. missense_variant = variant leads to change in amino acid sequence. inframe_deletion = deletions of 3, or multiples of 3, which remove whole codon sequences. intron_variant = variant occurs in intron. splice_region_variant = variant in splice site, may lead to splicing error in final mRNA transcript. stop_gained = variant results in premature stop codon in mRNA transcript. 3_prime_UTR_variant = variant occurs in 3" untranslated region (UTR)
	Impact	Impact of variant on normal gene function
	Gene_Symbol	Gene name
	Gene_ID	NCBI gene ID number
	Feature	NCBI transcript identifier
	All_Features	List of all NCBI transcript identifiers associated with the variant
	HGVSC	HGVSC variant name. Describes position and variant change in gene (nucleic acid) sequence in Feature
	HGVSP	HGVSP variant name. Describes position and variant change in protein (amino acid) sequence in Feature
	Transcript	The VEP annotated transcript name (e.g., NM_001127500.3)
	c_dot	VEP HGVSc annotation with transcript name removed (e.g., `c.3912C>T`)
	p_dot	VEP HGVSp annotation with transcript removed (e.g., `p.Asp1304=`)
	Exon	Affected exon number in Feature out of total number of exons. Applies to variants within exon regions only. (affected exon # / total # of exons)

Sheet Tab	Column Name	Definition/Description
	Intron	Affected intron number in Feature out of total number of introns. Applies to variants within intron regions only. (affected intron # / total # of introns)
	CDS_Position	cDNA sequence position in Feature
	Protein_Position	Affected amino acid number in Feature
	Amino_Acids	Amino acid resulting from variant (reference amino acid / variant amino acid)
	Codons	Position in codon where variant occurs and resulting codon with variant
	Co-located_Known_Variation	Variants that occur at the same position in publicly available databases
	COSMIC_LINK	URL link to the COSMIC database for the called variant
	Strand	Transcribed strand. -1 = negative strand, 1 = positive strand
	Repeat	Number of repeat bases, including repeat sequence (if applicable). i.e. 14G indicates a repeat of 14 Gs
	HGVS_Offset	Correction factor to synchronize genomic base position for indels in repeat regions in negative strand genes. Read alignment uses left-align paradigm (left most base assumed to be indel; first genomic position) while HGVS uses right-align paradigm (right most base assumed to be indel; first cDNA position)
	SIFT	Output from SIFT (v5.2.2). PREDICTION (SIFT score). Predicts impact of variant on protein function. Prediction possibilities: tolerated, deleterious. SIFT score is a range from 0 - 1. Scores > 0.05 are tolerated, scores of < 0.05 are deleterious
	POLYPHEN	Output from PolyPhen (v2.2.2). PREDICTION (PolyPhen score). Predicts impact of variant on protein function. Prediction possibilities: benign, possibly damaging, probably damaging. PolyPhen score is a range from 0 (benign) - 1 (probably damaging) and is the probability of variant leading to a damaging mutation. Benign = variant unlikely to cause damage to protein function. Possibly damaging = variant likely to cause damage to protein function but prediction is with low confidence. Probably damaging = variant likely to cause damage to protein function and prediction is with high confidence.
	AF	Allele frequency of variant found in global population (1000 Genomes version Phase 3)
	AFR_AF	Allele frequency of variant found in African populations (1000 Genomes version Phase 3)

Sheet Tab	Column Name	Definition/Description
	AMR_AF	Allele frequency of variant found in American populations (1000 Genomes version Phase 3)
	EAS_AF	Allele frequency of variant found in East Asian populations (1000 Genomes version Phase 3)
	EUR_AF	Allele frequency of variant found in European populations (1000 Genomes version Phase 3)
	SAS_AF	Allele frequency of variant found in South Asian populations (1000 Genomes version Phase 3)
	gnomAD_AF	Allele frequency of variant found in global population (gnomAD version r2.1)
	gnomAD_AFR_AF	Allele frequency of variant found in African/African American populations (gnomAD version r2.1)
	gnomAD_AMR_AF	Allele frequency of variant found in Latino/Admixed American populations (gnomAD version r2.1)
	gnomAD_ASJ_AF	Allele frequency of variant found in Ashkenazi Jewish populations (gnomAD version r2.1)
	gnomAD_EAS_AF	Allele frequency of variant found in East Asian populations (gnomAD version r2.1)
	gnomAD_FIN_AF	Allele frequency of variant found in European (Finnish) populations (gnomAD version r2.1)
	gnomAD_NFE_AF	Allele frequency of variant found in European (non-Finnish) populations (gnomAD version r2.1)
	gnomAD_OTH_AF	Allele frequency of variant found in Other populations (gnomAD version r2.1)
	gnomAD_SAS_AF	Allele frequency of variant found in South Asian populations (gnomAD version r2.1)
	CLIN_SIG	Clinical significance (ClinVar v201912)
	PiVAT_ClinSig	PiVAT clinical significance based on CLIN_SIG and IMPACT
Filtered False Positives	This sheet reports all false positive variants that do not pass PiVAT's filtering parameters.	
Overall Stats	Total Reads	Total number of sequencing reads for the library
	Overall:Q=30	Percentage of bases with Q score greater than or equal to 30
	Overall:Q=20	Percentage of bases with Q score greater than or equal to 20

Sheet Tab	Column Name	Definition/Description
	Properly Paired Read	Number of read mates properly paired, from paired end sequencing
	Properly Paired Read (%)	Percentage of total reads properly paired
	Mapped Reads	Percentage of total reads that map to human genome (hg19)
	Mapping Rate (%)	Percentage of total reads that map to human genome (hg19)
	On Target Reads	Reads that map to target amplicon regions of interest (ROIs)
	On Target Rate (%)	Percentage of mapped reads that map to target amplicon regions of interest (ROIs)
	Insert Size Mean	Mean size of the library insert (varies based on panel)
	Insert Size Median	Median size of the library insert
	Insert Size Std Dev	Standard deviation of library insert size
	Coverage_Mean	Mean base coverage of all bases within the defined ROI; with paired end sequencing, merged paired-end reads (forward and reverse) create a coverage of 1x
	STDEV	Standard deviation of mean base coverage
	Coverage_Median	Median base coverage of all bases within the defined ROI; with paired end sequencing, merged paired-end reads (forward and reverse) create a coverage of 1x
	Coverage_Max	Maximum base coverage of all bases within the defined ROI
	Coverage_Min	Minimum base coverage of all bases within the defined ROI
	Total_Number_Of_Reads	Total number of reads on target for all amplicons
	Total_Valid_Reads	Total number of reads contributing to paired end assembly after filtering
	On_Target_Ratio	The ratio between Total_Valid_Reads and Total_Number_Of_Reads. On_Target_Ratio = (Total_Valid_Reads / Total_Number_Of_Reads)
	Base_Coverage_Depth_>_(Nx)	Percent of bases that have a minimum base coverage greater than or equal to Nx (absolute coverage)
	Base_Coverage_Depth_>_(Nx)_ Relative_to_Mean_Coverage	Percent of bases that obtain at least Nx*mean base coverage, usually described as percent of mean base coverage. Used to determine uniformity of base coverage across ROIs in panel. Value of 100 indicates 100% of bases in all ROI are above the given Nx relative to the Coverage_Mean.

Sheet Tab	Column Name	Definition/Description
Segment Coverage	This sheet reports the Coverage_Mean, Coverage_Min and STDEV of each amplicon ROI for all samples initially added to the analysis. Additional columns include Target_Name (amplicon ROI identifier), Region (ROI segment position), Segment_Size (base pair size of ROI segment), and GC_Content_(%) (percentage of GC content within ROI segment).	

Appendix B: Microsatellite Instability (MSI) Caller

Microsatellite instability (MSI) is measured using MSIsensor (https://github.com/ding-lab/msisensor) in conjunction with Pillar's custom MSI46 panel. This software compares the distribution of read lengths at 53 microsatellite (MS) sites in the tumor genome with the same sites in a matched normal reference. MSI status (instable—MSI-high, or stable—MSS) is determined by the number of sites which differ.

MSI Sample Setup

The user must provide a matched normal sample for use as a reference in determining each microsatellite site's baseline read length distribution. Ideally this sample should be prepared with the same methodology and sequenced in parallel with its corresponding tumor sample.

MSI Analysis Setup

This section provides specific instructions to start an MSI analysis. Please refer to

Start Analysis section on page 14 for general instructions on how to start a PiVAT analysis.

1. Select **Start Analysis** on the left menu bar.
2. From the Start Analysis: 1. Start From page select the **MSI46** panel from the drop-down menu and select the desired samples to include in analysis.
3. Select **NEXT** to proceed.
4. From the Start Analysis: 2. Edit Definition page enter desired **Analysis Name**.
5. The samples you have selected will appear in the table as shown below.

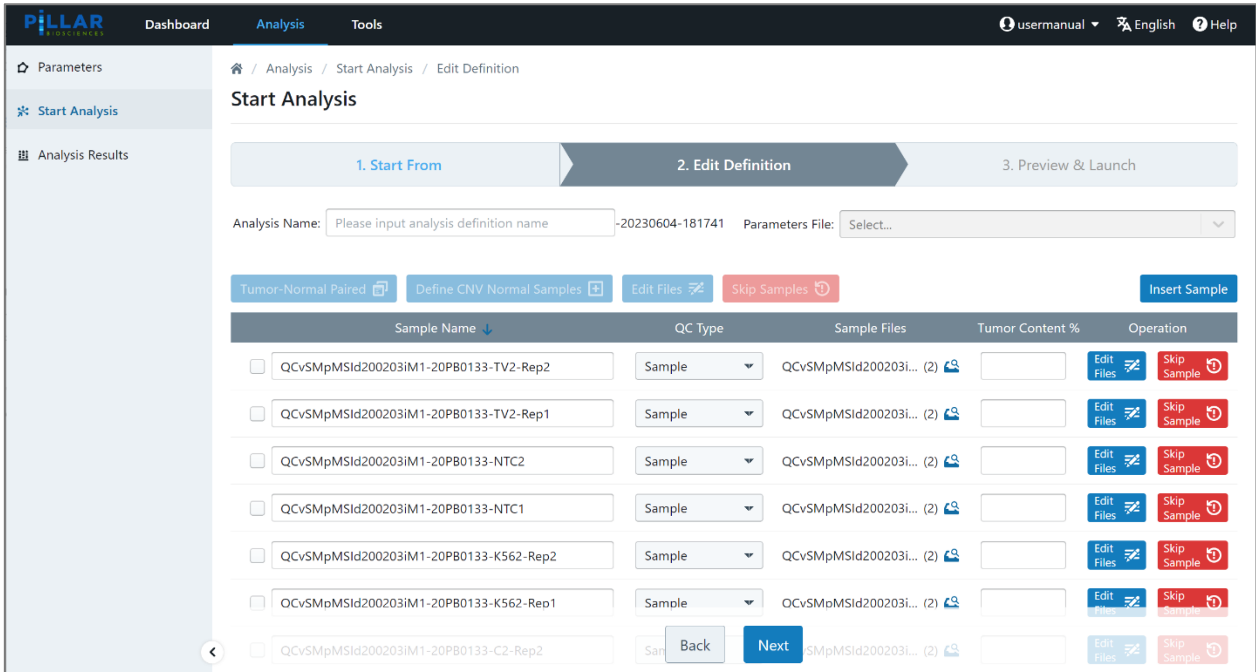


Figure 32 Start Analysis: 2. Edit Definition page – MSI Analysis

6. For each tumor/normal pair, **(A)** check the boxes to the left of the Sample Name in the table and **(B)** select the **Tumor Normal Paired** button. This will link the two input files and they will appear at the bottom of the table. Ensure that the files are defined correctly. **(C)** The definitions can be swapped by clicking the button to the left of the files.

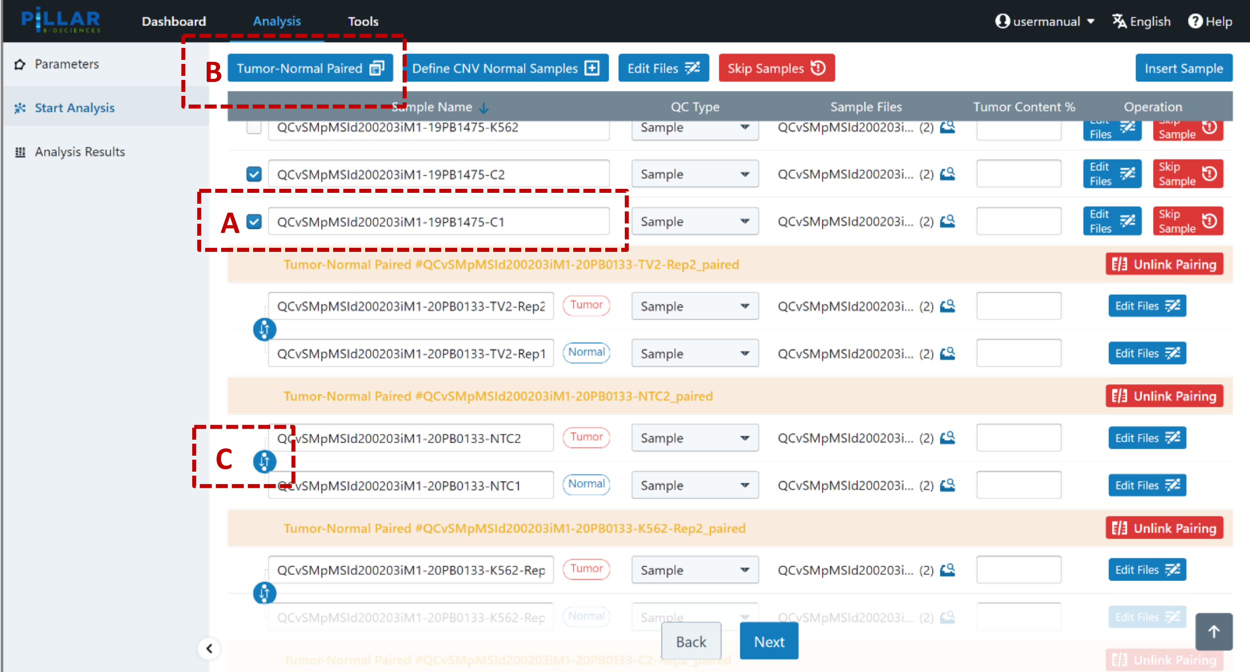


Figure 33 Start Analysis: 2. Edit Definition page – define tumor/normal sample pair

7. Once all the samples have been paired select **NEXT**.

8. Review the analysis input and select Launch Analysis to begin the run. The task status may now be monitored on the PiVAT Dashboard.

PiVAT Output: MSI_RESULTS file

Definition and/or description of result columns reported in MSI_RESULTS file sheet tabs are provided below.

Sheet Tab	Column Name	Definition/Description
MSI Call Report	**Tumor_Sample**	Unique Sample ID for each tumor sample in a matched pair
	Normal_Sample	Unique Sample ID for each normal sample in a matched pair
	Tumor_Sample_Type	The sample type associated with the tumor sample from a matched pair
	Normal_Sample_Type	The sample type associated with the normal sample from a matched pair
	MSI_Call	Whether the tumor sample exhibits microsatellite instability (True = MSI-High, False = MSS)
	Total_Number_of_Sites	The number of MS sites assessed for a tumor/normal pair
	Number_of_Somatic_Sites	The number of MS sites that are different between the tumor and the matched normal sample
	Somatic_Sites_Ratio	The ratio of differing MS sites to total MS sites assessed
Read Count Distribution	**Tumor_Sample**	Unique Sample ID for each tumor sample in a matched pair
	Normal_Sample	Unique Sample ID for each normal sample in a matched pair
	Tumor_Sample_Type	The sample type associated with the tumor sample from a matched pair
	Normal_Sample_Type	The sample type associated with the normal sample from a matched pair
	Target_Name	The amplicon ID covering the MS site
	Chromosome	The chromosome the MS site is located on
	MSI_Start	The location of the MS start site (hg19)
	Left_Flank	The 5-bp sequence to the left (upstream) of the MS site
	Repeat_Unit_Bases	The reference number of repeats and the [repeating sequence unit] comprising the MS site
	Right_Flank	The 5-bp sequence to the right (downstream) of the MS site
	Number_of_Repeat_Units	The number of possible repeating units (1-100)
	Tumor_Read_Count	The number of reads in the tumor sample with a repeat length specified by **Number_of_Repeat_Units** field
	Normal_Read_Count	The number of reads in the normal sample with a repeat length specified by **Number_of_Repeat_Units** field
Somatic Sites	**Tumor_Sample**	Unique Sample ID for each tumor sample in a matched pair
	Normal_Sample	Unique Sample ID for each normal sample in a matched pair

Sheet Tab	Column Name	Definition/Description
	Tumor_Sample_Type	The sample type associated with the tumor sample from a matched pair
	Normal_Sample_Type	The sample type associated with the normal sample from a matched pair
	Target_Name	The amplicon ID covering the MS site
	Chromosome	The chromosome the MS site is located on
	MSI_Start	The location of the MS start site (hg19)
	Left_Flank	The 5-bp sequence to the left (upstream) of the MS site
	Repeat_Times	The reference number of repeats of the repeating sequence unit
	Repeat_Unit_Bases	The bases comprising the repeating unit
	Right_Flank	The 5-bp sequence to the right (downstream) of the MS site
	Difference	The difference score (0-1) assigned to the MS site between tumor and normal
	P_Value	The p-value associated with the difference score
	FDR	The FDR-adjusted p-value associated with the difference score
	Rank	The rank, by p-value, of the MS site
Germline Sites	**Tumor_Sample**	Unique Sample ID for each tumor sample in a matched pair
	Normal_Sample	Unique Sample ID for each normal sample in a matched pair
	Tumor_Sample_Type	The sample type associated with the tumor sample from a matched pair
	Normal_Sample_Type	The sample type associated with the normal sample from a matched pair
	Target_Name	The amplicon ID covering the MS site
	Chromosome	The chromosome the MS site is located on
	MSI_Start	The location of the MS start site (hg19)
	Left_Flank	The 5-bp sequence to the left (upstream) of the MS site
	Repeat_Times	The reference number of repeats of the repeating sequence unit
	Repeat_Unit_Bases	The bases comprising the repeating unit
	Right_Flank	The 5-bp sequence to the right (downstream) of the MS site
	Genotype	The repeat lengths of alleles in the Normal sample

Appendix C: Somatic CNV Caller

There are two copy number variation (CNV) callers implemented within PiVAT, to handle FFPE and cfDNA panels respectively. The FFPE CNV calls are reported at gene- and exon-levels, cfDNA calls are currently restricted to gene-level only. One CNV call is displayed as one row in the report. All amplicons of the panel are used in the CNV analysis.

CNV Sample Setup

FFPE CNV caller: For the best CNV caller performance, **3 to 5 (minimum 2) normal samples** to be used as normalization reference is recommended. These normal samples should have similar sample condition and preparation process as the potentially positive samples. See section below for recommended setting for CNV analysis.

cfDNA CNV caller: The cfDNA CNV caller requires at least one CNV normal sample, with the normal samples having similar sample condition and preparation process as the potentially positive samples. The quality of the calls improves with increasing numbers of normal samples.

CNV Analysis Setup

This section provides specific instructions to start a CNV analysis. Please refer to

Start Analysis section on page 14 for general instructions on how to start a PiVAT analysis.

1. Select Start Analysis on the left menu bar.
2. From the Start Analysis: 1. Start From page, select a desired CNV panel from the drop-down menu and select the desired samples to include in analysis.
3. Select Next to proceed.
4. From the Start Analysis: 2. Edit Definition page, enter desired **Analysis Name**.
5. The samples you have selected will appear in the table as shown below.
6. To define normal samples, **(A)** check the boxes to the left of the Sample Name in the Edit Definition table and **(B)** select the **Define CNV Normal Samples** button.
7. If using the **cfDNA CNV Caller** (currently only applies to the P-LBX-01 panel): **Tumor Content %** should be specified if known. In the **Tumor Content %** column enter a value from 1e-8 to 100, representing the tumor content as a percentage for the sample on that row. If this is left empty the tumor content will either be inferred or given a default (**CNV_DEFAULT_TUMOR_CONTENT_LOWER, CNV_DEFAULT_TUMOR_CONTENT_UPPER** may be overridden when defining a Custom Parameter for P-LBX-01).

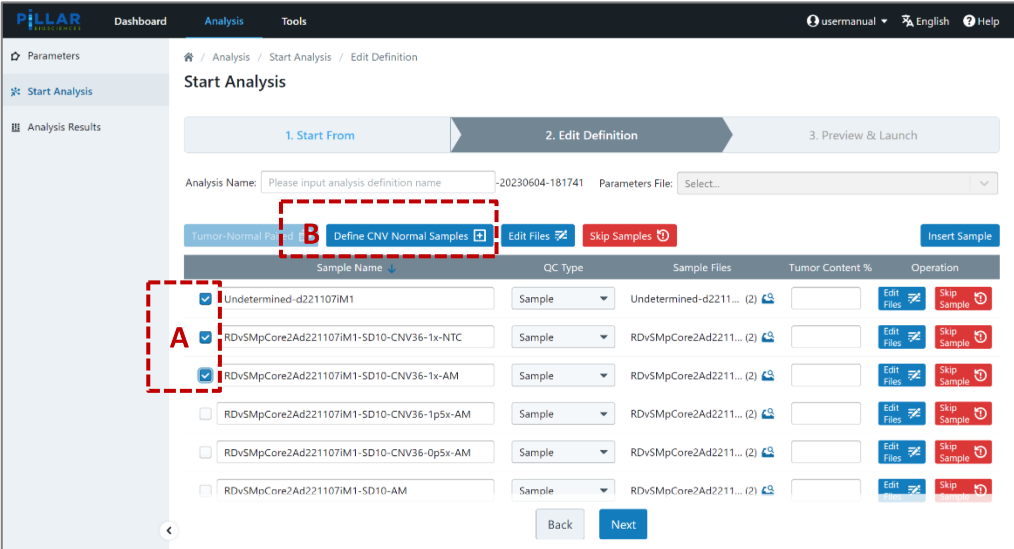


Figure 34 Start Analysis: 2. Edit Definition page – define CNV normal sample(s)

8. The selected samples will be grouped as **Normal Samples**. Samples can be added or removed from the list of Normal Samples using the same **Define CNV Normal Samples** button or the **Undefine Normal Sample** buttons.

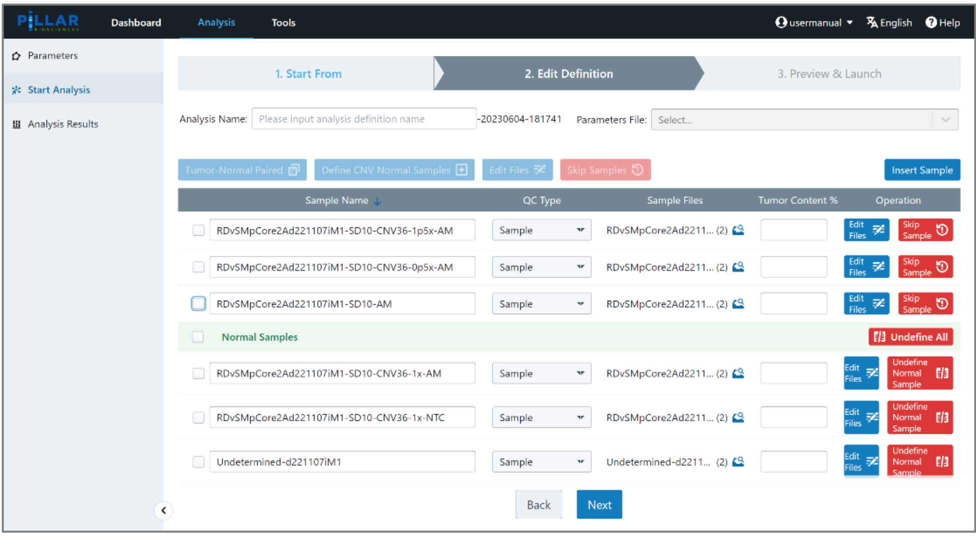


Figure 35 Start Analysis: 2. Edit Definition page – Normal Samples group

9. **For FFPE panels:** If no normal negative reference samples are provided, at least 10 potentially positive samples are required to run CNV analysis. In this setting, the CNV analysis will run with center percentile normalization algorithm. The sample setting configuration will be automatically detected by PiVAT. No user parameter adjustment is needed. In addition, the number of samples with the same CNV type cannot exceed 30% of all samples. We recommend pooling samples with different CNVs (CNVs with different exons/genes/lengths) in this setting.

10. For PiVAT 2023.1, there is a post-call CNV filter to filter out CNV calls that fail to pass the gene specific thresholds set in the Post-Filtering section of Analysis Parameters (see page 12). Each CNV panel is designed

with a specific set of genes the panel targets, with thresholds for confident CNV calls on those genes. The default values can be seen in the same Post-Filtering section when creating a new Analysis Parameter.

11. The normalized copy number ratio can be found in 'Normalized Coverages' sheet in CNV_RESULTS excel file. Note that the "Copy Number Ratio" in PiVAT is defined as the copy number ratio of a potentially positive sample to that of the negative reference samples (diploid with 2 copy number).

CNV QC parameters: FFPE panels

The following is a list of user adjustable QC parameters and default values in ( ).

1. **CNV_ALLOW_FEWER_THAN_MIN_NUM_SAMPLES (False)**

   This parameter allows running with fewer than 10 samples and no negative reference samples. The default value is "False". When the parameter is toggled to True, CNV analysis still needs at least 3 samples. Only toggles on for intended low sample number run mode if number of total remaining samples are between 3 and 10 and number of negative samples is less than 2.

2. **CNV_QC_ZERO_COVERAGE_PCT_THRESHOLD_NORMAL_SAMPLE (0.5)**

   Percentage of zero coverage targets needed for a negative reference sample to pass the negative reference QC filter. The default value is 0.5, which means a negative reference sample passes the negative reference QC filter if less than 50% of its targets have zero coverage.

3. **CNV_QC_ZERO_COVERAGE_PCT_THRESHOLD_POS_SAMPLE (0.5)**

   Percentage of zero coverage targets needed for a potentially positive sample to pass the sample QC filter. The default value is 0.5, which means a sample passes the sample QC filter if less than 50% of its targets have zero coverage.

4. **CNV_QC_ABSOLUTE_COVERAGE_THRESHOLD_NORMAL_SAMPLE (50)**

   Mean segment coverage needed for a negative reference sample to pass the negative reference QC filter. The default value is 50, which means a negative reference sample should have the mean segment coverage >= 50 to pass the negative reference QC filter.

5. **CNV_QC_ABSOLUTE_COVERAGE_THRESHOLD_POS_SAMPLE (10)**

   Mean segment coverage needed for a potentially positive sample to pass the sample QC filter. The default value is 10, which means a sample should have the mean segment coverage >= 10 to pass the sample QC filter.

6. **CNV_QC_RELATIVE_COVERAGE_PCT_THRESHOLD_NORMAL_SAMPLE (0.1)**

   Minimum mean segment coverage percentage compared to other samples' mean segment coverage needed for a negative reference sample to pass the negative reference QC filter. The default value is 0.1, which means a negative reference sample should have the mean segment coverage >= 10% of other normal samples' mean segment coverage to pass the negative reference QC filter.

7. **CNV_QC_RELATIVE_COVERAGE_PCT_THRESHOLD_POS_SAMPLE (0.1)**

   Minimum mean segment coverage percentage compared to other samples' mean segment coverage needed for a potentially positive sample to pass the sample QC filter. The default value is 0.1, which means a sample should have the mean segment coverage >= 10% of the CNV normal samples' mean segment coverage to pass the sample QC filter.

8. **CNV_QC_LOW_COVERAGE_PCT_THRESHOLD_NORMAL_SAMPLE (0.1)**

   Percentage of low coverage targets needed for a negative reference sample to pass the negative reference QC filter. The default value is 0.1, which means a negative reference sample should have <= 10% of low coverage targets to pass the negative reference QC filter.

9. **CNV_QC_Q30_THRESHOLD (80)**

   Minimum threshold for Quality 30 for both normal and potentially positive samples to pass the QC filter. The default value is 80, which means a sample (positive or negative) should have overall Q30 >= 80 to pass the sample QC filter.

10. **CNV_QC_ON_TARGET_RATE_THRESHOLD (90)**

    Minimum On Target Rate threshold for both negative and potentially positive samples to pass the QC filter. The default value is 90, which means a sample (positive or negative) should have an On Target Rate >= 90 to pass the sample QC filter.

11. **CNV_QC_CORRELATION_COEFFICIENT_THRESHOLD_NORMAL_SAMPLE (0.5)**

    Minimum correlation coefficient of a negative reference sample with the rest of other negative reference samples for that sample to pass the negative reference sample QC filter. The default value is 0.5, which means a negative reference sample should have a correlation coefficient >= 0.5 to pass the negative reference QC filter.

12. **CNV_QC_CORRELATION_COEFFICIENT_THRESHOLD_POS_SAMPLE (0.2)**

    Minimum correlation coefficient of a potentially positive sample with the negative reference samples for that sample to pass the sample QC filter. The default value is 0.2, which means a sample should have a correlation coefficient >= 0.2 to pass the sample QC filter.

13. **CNV_QC_OUTLIER_CENTER_PERCENTILE_POS_SAMPLE (40)**

    The center percentile is used to compare the correlation and relative coverage for potentially positive samples. If CNV normal samples are provided, the center percentile method is not used. Otherwise, the correlation and relative coverage is calculated against the center samples for each amplicon.

cfDNA CNV Caller QC parameters:

The following is a list of user adjustable QC parameters and default values in ( ).

1. **CNV_MINIMUM_AMPLICON_COVERAGE (5000)**

   Minimum amplicon coverage below which to exclude amplicons from CNV analysis. Values are integers from 0 to 1e12.

2. **CNV_MINIMUM_KEPT_AMPLICON_PROPORTION (0.8)**

   If fewer than this proportion of amplicons survive QC fail this sample/normal pair with `Amplicon Drop Out`. Values are floats from 0.0 to 1.0.

cfDNA CNV Caller Model Parameters:

We have provided substantial flexibility to control nearly every aspect of the cfDNA CNV Caller. However, these parameters should only be modified with the help of Pillar support, requiring familiarity with Bayesian nonparametric statistics and inference, and experience with the Pillar cfDNA CNV Caller.

3. **CNV_CLUSTER_ALPHA (0.01)**

   The concentration parameter of the base measure, with larger values leading to more high probability clusters. Values are floats between 1e-5 and 1.0.

4. **CNV_DEFAULT_TUMOR_CONTENT_LOWER (1.0)**

   When no user-specified tumor content is provided, this value is used as the lower bound for sample tumor content percentage. This will be used only when the Tumor Content (%) field in Edit Definition is empty. Values are floats, representing percentages, from 1e-6 to 100.0.

5. **CNV_DEFAULT_TUMOR_CONTENT_UPPER (30)**

   When no user-specified Tumor Content (%) is provided, this value is used as the upper bound for sample tumor content percentage. This will be used only when the Tumor Content (%) field in Edit Definition is empty. Values are floats, representing percentages, from 1e-6 to 100.0, and should be set to be larger than **CNV_DEFAULT_TUMOR_CONTENT_LOWER.**

6. **CNV_AUTO_INFER_SAMPLE_TUMOR_CONTENT (False)**

   Whether to automatically infer the expected tumor content from in-sample SNP/Indel calls. **Experimental**.

7. **CNV_USE_METADATA_SAMPLE_TUMOR_CONTENT (True)**

   Whether to use user-specified sample Tumor Content (%) in Edit Definition, when provided. Possible values are True and False, with True indicating that user-specified sample Tumor Content (%) will be used.

8. **CNV_METADATA_SAMPLE_TUMOR_CONTENT_LOWER_FACTOR (0.8)**

   This factor is multiplied by the Tumor Content (%) provided in Edit Definition to create the lower bound estimate for sample tumor content percentage. Values are floats. Only applies for samples that have user-defined Tumor Content (%) specified in Edit Definition.

9. **CNV_METADATA_SAMPLE_TUMOR_CONTENT_UPPER_FACTOR (1.2)**

   This factor is multiplied by the Tumor Content (%) provided in Edit Definition to create the upper bound estimate for sample tumor content percentage. Values are floats. Only applies for samples that have user-defined Tumor Content (%) specified in Edit Definition.

10. **CNV_MAXIMUM_NUMBER_OF_COPY_NUMBER_CLUSTERS (10)**

    The maximum number of CNV clusters to fit. Values are integers >= 3.

11. **CNV_CLUSTER_COPY_NUMBER_DISPERSION (1.0)**

    The spread (e.g., variance) parameter of the cluster copy number distribution. Only has an effect when CNV_FIXED_COPY_NUMBER is False. For the Poisson distribution this is the mean * CNV_CLUSTER_COPY_NUMBER_DISPERSION, set by an affine transformation. For the GammaPoisson distribution this is the rate parameter, set directly. Values are floats.

12. **CNV_CLUSTER_COPY_NUMBER_CONCENTRATION (2.0)**

    The concentration parameter (e.g., mean) of the cluster copy number distribution. Only has an effect when CNV_FIXED_COPY_NUMBER is false. For the Poisson distribution this is the mean. For the GammaPoisson distribution this is the shape parameter, set directly. Values are floats.

13. **CNV_SORT_INITIAL_VALUES (False)**

    Whether or not to sort the initial values for the copy number clusters. Values are True or False, with sorting enabled when True. Only applies when CNV_INIT_TO_2 and CNV_INIT_TO_UNIFORM are False.

14. **CNV_AMPLICON_NOISE_DISPERSION (1.0)**

    Control the spread of background amplicon centered log copy number ratio noise estimates. Smaller values mean less variation. Values are non-negative floats. Only has an effect when CNV_FIX_NOISE is False.

15. **CNV_CLUSTER_COPY_NUMBER_DISTRIBUTION (‘GammaPoisson’)**

    Base measure of copy number. Possible values are ‘Poisson’ and ‘GammaPoisson’ corresponding to a Poisson distribution and the compound Gamma Poisson distribution respectively. This is the distribution from which each cluster tumor copy number is drawn. The ‘GammaPoisson’ option allows for higher variance compared to ‘Poisson’, making large copy numbers more likely possibilities.

16. **CNV_FIX_TC (True)**

    Whether to treat estimated tumor content as fixed or infer tumor content. Setting CNV_FIX_TC to False allows for more Bayesian characterization of uncertainty in the call, resulting in wider credible intervals as opposed to a (fixed) point estimate. Possible values are True and False.

17. **CNV_FIX_NOISE (True)**

    Whether to treat observational noise as fixed. It is strongly recommended that this parameter be set to True. Setting to False can result in excessive shrinkage by the Dirichlet process resulting in a single cluster (no calls). Possible values are True and False.

18. **CNV_FIX_CLUSTER_COPY_NUMBER (False)**

    Whether to treat tumor DNA copy number as fixed. When True, tumor DNA copy number will be taken directly from initial values, and should only be used when CNV_INIT_TO_2 is False, as otherwise all tumor copy numbers will be trivially 2. Possible values are True and False.

19. **CNV_MAX_COPY_NUMBER (202.0)**

    The maximum possible tumor DNA copy number that the cfDNA CNV Caller is allowed to infer. The tumor DNA copy number is the idealized copy number in tumor cells (as opposed to the sample tumor copy number, which is the result of a mixture of normal and tumor DNA). In low

tumor content settings, this parameter, alongside the provided tumor content tunes signal to noise ratio. Values are floats and must be >= CNV_MIN_COPY_NUMBER.

20. **CNV_MIN_COPY_NUMBER (1.6)**

    The minimum tumor DNA copy number. Values close to 2 prevent calling copy number deletions , but improve the ability to detect copy number amplifications. Values are floats.

21. **CNV_DISCRETE_HMC_GIBBS (True)**

    Whether to use a Discrete Gibbs HMC MCMC kernel as opposed to a standard kernel. This results in slower sampling but has desirable theoretical/numerical properties. Possible are True and False, with the Discrete Gibbs HMC MCMC kernel enabled when True.

22. **CNV_PARAMETER_STRATEGY (‘median’)**

    This defines the averaging strategy of the posterior samples. The default, ‘median’, is robust to outliers. Possible values are ‘median’, ‘mean’, and ‘mode’.

23. **CNV_GENE_STRATEGY (‘mean’)**

    Strategy for averaging amplicons within a gene into a gene-level sample copy number ratio estimates. Possible values are ‘median’, ‘mean’, and ‘mode’.

24. **CNV_TUMOR_CONTENT_STRATEGY ('median')**

    Strategy for averaging tumor content estimates across the MCMC samples. Only important if tumor content is inferred rather than fixed. Possible values are 'median', 'mean', and 'mode'.

25. **CNV_AGGREGATION_STRATEGY ('mean')**

    Strategy for performing within-sample parameter aggregation from the ensemble of individual putative case / CNV Normal fits. Only applies when more than 1 CNV Normal is provided. Possible values are 'median', 'mean', 'min', 'max'.

26. **CNV_SEED (2023)**

    The random number generator seed, to ensure reproducibility. Values are integers.

27. **CNV_GENE_SMOOTHING_WEIGHT (0.7)**

    The amount of count averaging within a gene. 0 correspond to no averaging (raw counts for each amplicon) while 1.0 corresponds to every amplicon within the gene having identical counts (average counts). Values are floats from 0.0 to 1.0.

28. **CNV_CONSIDER_TOP_N_GENE_CALLS (20)**

    How many of the top (sorted in descending order) amplicon copy number values to consider when averaging amplicons within a gene to generate the gene copy number ratio call. Values are integers from 0 to 100,000.

29. **CNV_SHOW_NEUTRAL_CALLS (True)**

    Whether or not to report genes that are below the Gain or Loss copy number ratio threshold defined for those genes. See page 13 (CNV Threshold Grid) for details on how to edit these thresholds through the PiVAT webapp. When **CNV_SHOW_NEUTRAL_CALLS** is True, genes with inferred copy number ratios below the copy number ratio threshold will appear in the CNV Calls sheets of CNV_RESULTS and CUSTOMER_REPORT, with the label "Neutral" in the Relative Call Gain(Loss) column. Possible values are True and False.

30. **CNV_INIT_TO_UNIFORM (False)**

    Instead of initializing the Cluster Copy Number to random values between 2 and CNV_MAX_COPY_NUMBER, initialize them to a uniformly distributed random point in the support of the Cluster Copy Number distribution. Possible values are True and False. Cannot be set at the same time as CNV_INIT_TO_2 or CNV_INIT_TO_VALUES.

31. **CNV_INIT_TO_2 (True)**

    Initialize all Cluster Copy Number values to 2. Possible values are either True or False. Cannot be set at the same time as CNV_INIT_TO_UNIFORM or CNV_INIT_TO_VALUES.

32. **CNV_INIT_TO_VALUES (None)**

    Initialize the Cluster Copy Number to values specified in this comma-separated list of numbers. Example: For 2 CNV_MAXIMUM_NUMBER_OF_COPY_NUMBER_CLUSTERS, `2,2` would initialize both clusters to 2. Values are strings of comma-separated integers or floating-point numbers, with as many values as specified CNV_MAXIMUM_NUMBER_OF_COPY_NUMBER_CLUSTERS. Cannot be set at same time as CNV_INIT_TO_UNIFORM or CNV_INIT_TO_2.

33. **CNV_AVERAGE_ALL_NORMALS (False)**

When multiple normal samples are selected, whether to average the amplicon coverages of those normals and fit the putative case samples against that averaged "normal", instead of fitting the putative case samples against individual normals and averaging the resulting parameter estimates. This results in cheaper computation time (single model fit rather than multiple for unaveraged normal) but can more easily overpolish. Possible values are True and False.

PiVAT Output: CNV_RESULTS file

Definition and/or description of result columns reported in CNV_RESULTS file sheets are provided below.

Sheet Tab	Column Name	Definition/Description
CNV Calls	**Sample ID**	Unique Sample ID for each sample.
	Gene ID	Simplified gene name for the called CNV segment.
	Copy Number Ratio	The ratio of the copy number of called CNV segment to the copy number (2) of a negative normal sample.
	Relative Gain/Loss	Relative Gain or Loss label of the called CNV segment. If the copy number ratio is more than 1, it is labeled as "Gain". Otherwise, it is labeled as "Loss".
	Std Dev	Standard deviation calculated from the copy number ratios of the amplicons in the called CNV segment.
	P-Value	P-value of the called CNV segment.
	Amplicon Count	Count of amplicons in the called CNV segment.
	CNV Start	The estimated start location of the called CNV segment.
	CNV End	The estimated end location of the called CNV segment.
	Exon List	The list of the exons in the called CNV segment. **Only reported for FFPE caller.**
CNV Run QC	**QC Criteria**	Indicate which QC criteria that each row is reporting, including "Negative_Reference", "Sample" and "Run_Status".
	QC Status	Whether the QC is passed for each QC criteria. If QC is passed, it is labeled as "Pass". If QC is failed, it is labeled as "Fail". The "Run_Status" is labeled with the actual setting that CNV analysis is run with unless the CNV run is completely failed, where it is labeled as "Fail".
Filtered CNV Samples	**Sample ID**	Unique Sample ID for each sample
	Filter Reason	The reason that the sample is filtered out from the CNV analysis.
	Sample Type	Indicate whether the reported sample is "Sample" or "Negative Reference". This is pre-defined by the user before starting the SA analysis.
CNV Segment Coverages	This sheet reports the raw segment coverages of each amplicon for all the samples initially added to the CNV analysis. The column headers are Amplicon Names and the row indices are Sample IDs.	

Sheet Tab	Column Name	Definition/Description
Normalized Coverages	This sheet reports the normalized copy number ratio of each amplicon for the samples that are included in the CNV analysis. Note that the filtered samples are not reported in this sheet. The column headers are the covered region of each amplicon ("Region") plus all the Sample IDs; the row indices are Amplicon Names.	

CNV Calls Table

The CNV Calls table contains the CNV calls for the sample(s) selected in **Select Sample.**

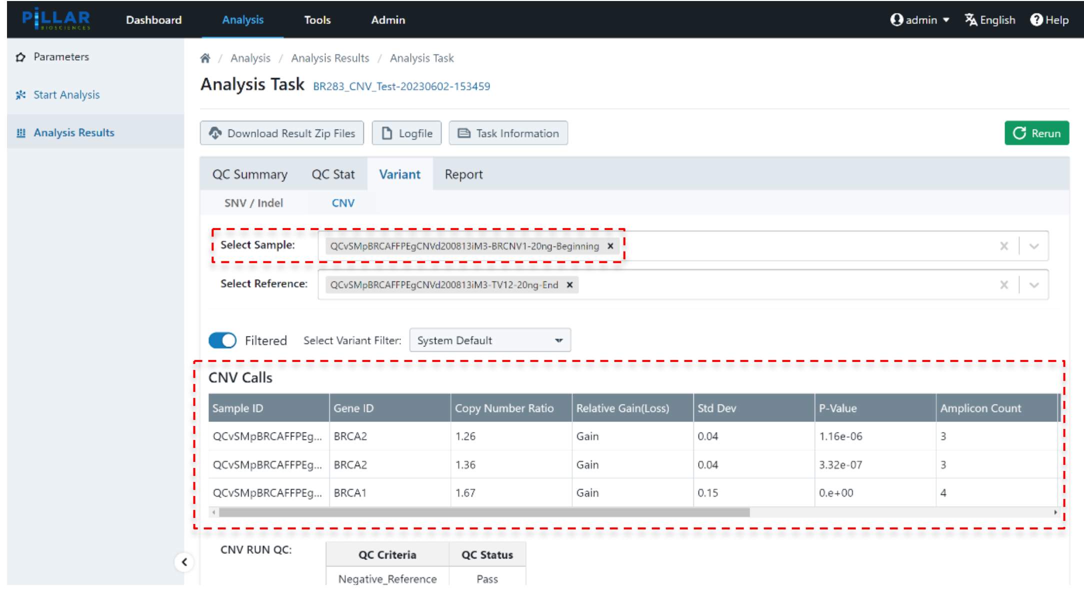


Figure 36 Analysis Results: CNV Calls Table

Definition and/or description of result columns reported in the CNV Calls table are provided below.

Column Name	Definition/Description
Sample ID	Unique Sample ID for each sample.
Gene ID	Simplified gene name for the called CNV segment.
Copy Number Ratio	The ratio of the copy number of called CNV segment to the copy number (2) of a negative normal sample.
Relative Gain/Loss	Relative Gain or Loss label of the called CNV segment. If the copy number ratio is more than 1, it is labeled as "Gain". Otherwise, it is labeled as "Loss".
Std Dev	Standard deviation calculated from the copy number ratios of the amplicons in the called CNV segment.

Column Name	Definition/Description
P-Value	**For FFPE caller:** P-value of the called CNV segment. **For cfDNA caller**: 0 if cfDNA CNV Caller considers the copy number to be non-neutral, or 1 otherwise.
Amplicon Count	Count of amplicons in the called CNV segment.
CNV Start	The estimated start location of the called CNV segment.
CNV End	The estimated end location of the called CNV segment.
Exon List	The list of the exons in the called CNV segment.

CNV Filtering

CNV Calls in the CNV Calls Table can be filtered using the **Filtered** toggle displayed below. If no custom parameter is assigned to this task, **System Default** is selected. If the task was run with a custom Parameter, that custom filter will be selected.

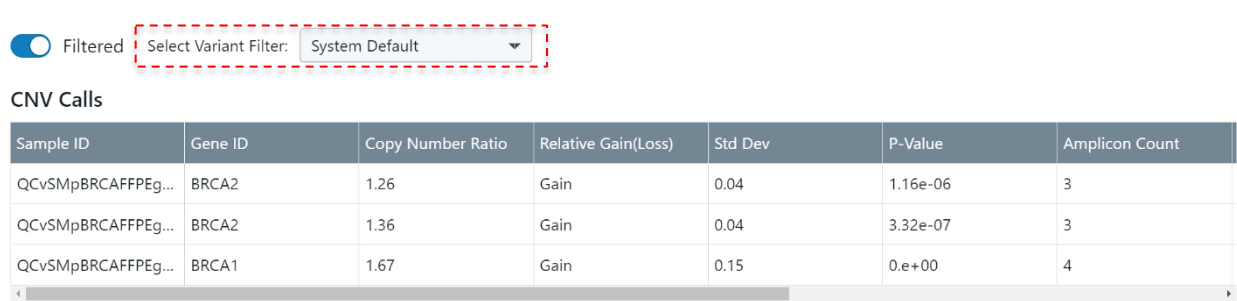


Figure 37 Analysis Results: CNV Call Filtering

Apply a custom filter preset by selecting it in the **Select Variant Filter** dropdown. The CNV Calls table will update to show only the CNV Calls that pass the selected filter. Clicking **Save** will assign the chosen filter to this task and **regenerate the PDF reports for this task.** Clicking **Discard** will switch to the selection to the filter that is assigned to the task.

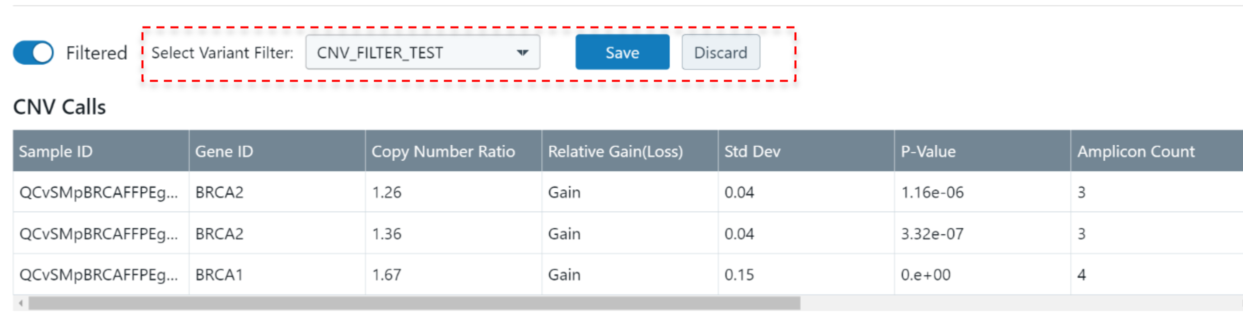


Figure 38 Analysis Results: CNV Call Filtering – Saving the new filter

If the Filtered toggle is off, all CNV calls will appear in the CNV Calls table with no filtering.

CNV Plot

A scatter plot will be displayed for each selected CNV sample. Each point represents the copy number ratio of a target.

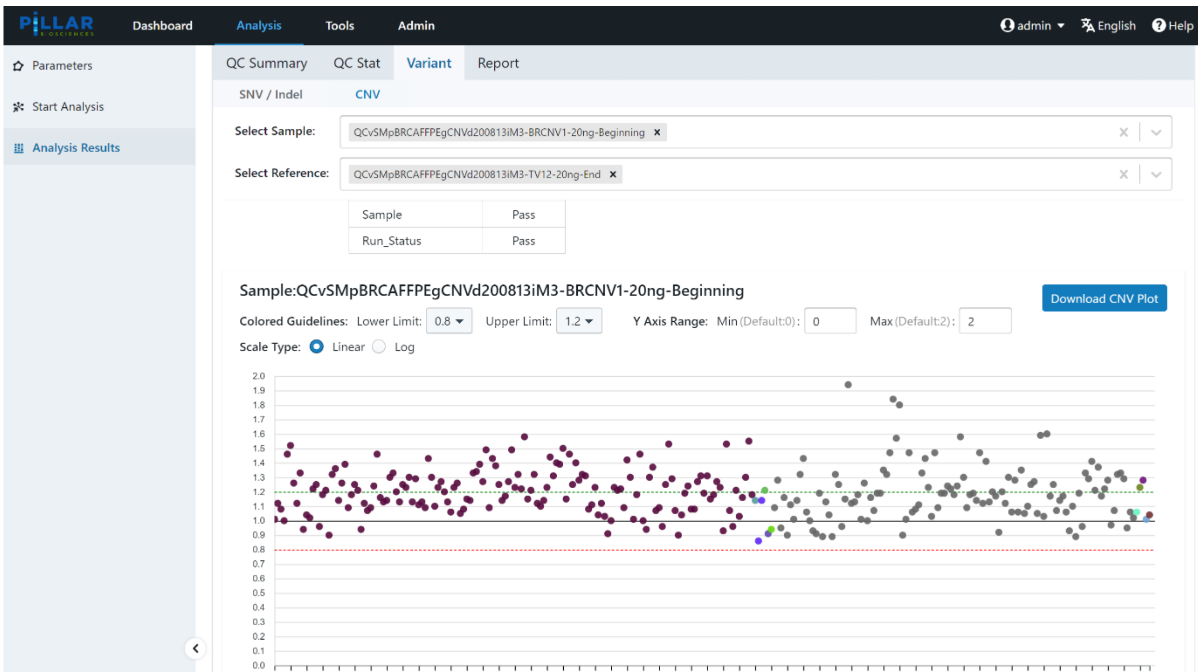


Figure 39 Analysis Results: CNV Plot

Genes can be toggled on/off by clicking the name of the gene in the legend. In the example below, the **BRCA1** gene has been hidden.

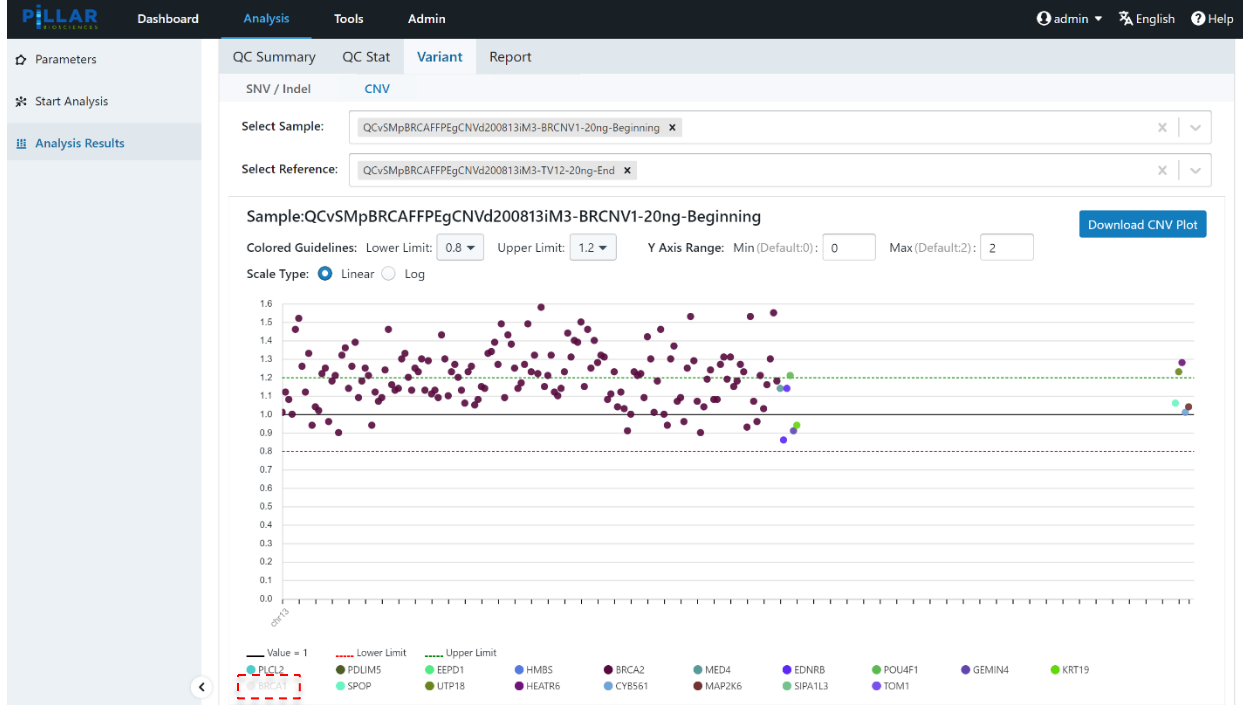


Figure 40 Analysis Results: CNV Plot – Toggle Gene

The plot can be saved as a PNG by clicking the blue **Download CNV Plot** button. Any change made to the plot will be reflected in the saved image.

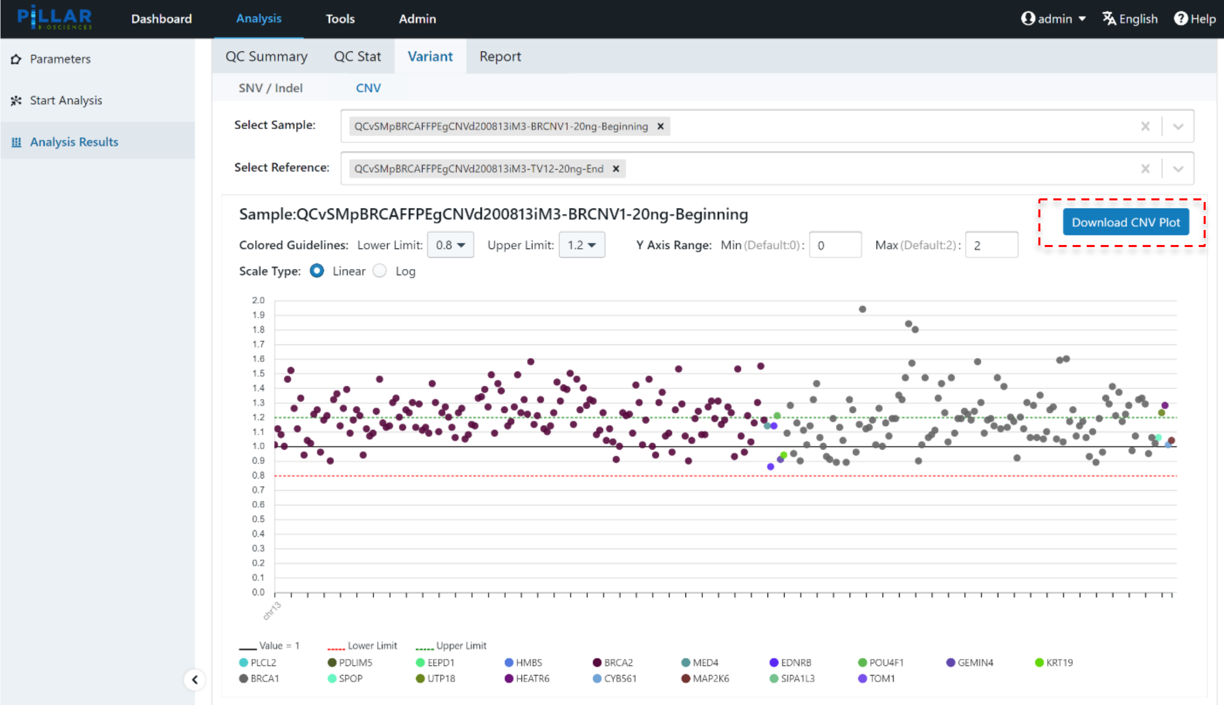


Figure 41 Analysis Results: CNV Sample Plot – Save Plot as PNG

Note that the plot shows the copy number ratios of all targets in the genes of interest for the panel. The data points on the plot do not get filtered out by the selected preset filter.

CNV Panel Specific Notes

Notes in this section pertain to specific panels. Any given note below can be ignored if your PiVAT installation does not include the particular panel.

oncoReveal LBx Core (P-LBX-01) utilizes the **cfDNA caller**. **P-LBX-01 requires at least 1 CNV Normal to be specified** (using Define CNV Normal Samples button in Edit Definition). If sample tumor content is known, it should be specified in the **Tumor Content %** column (as a percentage) for improved CNV calling performance.

For the oncoReveal Multi-Cancer with CNV v4 (HS341), if running cell-line or germline samples, it is recommended to create a custom Parameter to filter copy gain calls at 1.20, copy loss calls at 0.8, and amplicon count at 3 amplicons instead of the default values.

For the oncoReveal BRCA1 & BRCA2 plus CNV Panel (BR283), if running somatic samples, it is recommended to create a custom Parameter to filter copy gain calls at 1.20 and copy loss calls at 0.80 instead of the default values.

For the oncoReveal BRCA1 & BRCA2 plus CNV Panel (BR283), when viewing the CNV results in the CNV Calls table, there may be multiple CNV calls for the same gene and same exons. This is expected, as the panel was designed with both a gene caller and an exon-level cluster caller, and a copy gain or loss may be detected in multiple clusters within a given exon or gene. The CNV Start and CNV End positions can be used to distinguish the calls apart, and the custom Parameter filter can be used to isolate pertinent CNV calls.

Appendix D: Thalassemia CNV Caller

The Thalassemia analysis is based on the double normalization method, including one per-sample normalization and one per-amplicon normalization. The normalization baseline is calculated from negative reference samples. The copy number variance (CNV) is detected by amplicon clusters and the Thalassemia type of each sample is called by matching the edges of a CNV with the edges of a list of common Thalassemia types.

Thalassemia CNV Sample Setup

For each Thalassemia CNV analysis run, the user should provide 3-5 (minimum 2) in-run normal (negative) reference samples with similar sample condition and preparation process as the positive samples. If less than 2 negative reference samples are provided, the run will fail.

See CNV Sample Setup section in Appendix C: Somatic CNV Caller for instructions to define normal samples to be used as negative references.

Thalassemia CNV Analysis Setup

1. See CNV Analysis Setup section in Appendix C: Somatic CNV Caller for instructions to setup a Thalassemia CNV analysis.
2. The THAL_RESULTS excel file is output upon completion of the analysis.
3. Detailed Thalassemia call information are in the “Thalassemia Calls” sheet of the THAL_RESULTS excel file.
4. The fully normalized copy number ratios can be found in “Fully Normalized” sheet in THAL_RESULTS excel file. Note that the copy number ratio in PiVAT is defined as the copy number ratio of a potentially positive sample to that of the negative reference samples (diploid with 2 copy number).

Thalassemia CNV QC parameters

See CNV QC parameters section in Appendix C: Somatic CNV Caller for a list of user adjustable QC parameters.

PiVAT Output: THAL_RESULTS file

Definition and/or description of result columns reported in THAL_RESULTS file sheets are provided below.

Sheet Tab	Column Name	Definition/Description
Thalassemia Summary	**Sample ID**	Unique Sample ID for each sample.
	Thalassemia Call Status	Whether each sample is called as Thalassemia CNV positive by PiVAT. “TRUE” means a sample is Thalassemia CNV positive while “FALSE” indicates a sample is Thalassemia CNV negative.
	QC Status	Whether each sample passes the sample QC. Sample is labeled as “Pass” if passing sample QC and “Fail” if failing sample QC.
	Sample Type	Indicate whether a sample is defined as “Sample” or “Negative_Reference” by the user.
Thalassemia Calls	**Sample ID**	Unique Sample ID for each sample.
	Thal Type	The Thalassemia type detected by the “Thal Type Caller” for a sample.
	Copy Number Ratio	The ratio of the copy number of called Thalassemia CNV to the copy number (2) of a negative normal sample.
	P-Value	P-value of the called Thalassemia CNV.
	Relative Gain/Loss	Relative Gain or Loss label of the called CNV segment. If the copy number ratio is more than 1, it is labeled as “Gain”. Else (when the copy number ratio is less than 1) it is labeled as “Loss”.
	CNV Start Min	The estimated minimum start location of the called Thalassemia CNV.
	CNV Start Max	The estimated maximum start location of the called Thalassemia CNV.
	CNV End Min	The estimated minimum end location of the called Thalassemia CNV.
	CNV End Max	The estimated maximum end location of the called Thalassemia CNV.
Thalassemia Run QC	**QC Criteria**	Indicate which run QC criteria that each row is reporting, including “Negative_Reference”, “Sample” and “Run_Status”.
	QC Status	Whether the QC is passed for each run QC criteria. When run QC is passed, it is labeled as “Pass”. When run QC is failed, it is labeled as “Fail”.
THAL Filtered Samples	**Sample ID**	Unique Sample ID for each sample
	Filter Reason	The reason that the sample is filtered out from the Thalassemia analysis.
	Sample Type	Indicate whether the reported sample is “Sample” or “Negative_Reference”. This is pre-defined by the user before starting the SA analysis.

Sheet Tab	Column Name	Definition/Description
CNV Segment Coverages		This sheet reports the raw segment coverages of each amplicon for all the samples initially added to the Thalassemia analysis. The column headers are Amplicon Names and the row indices are Sample IDs.
Normalized Coverages		This sheet reports the normalized copy number ratio of each amplicon for the samples that pass the sample QC and are actually included in the Thalassemia analysis. Note that the filtered samples are not reported in this sheet. The column headers are Amplicon Names and the row indices are Sample IDs.

Thalassemia Type Calls Table

Thalassemia Type Calls table contains all calls for the sample(s) selected in **Select Sample.**

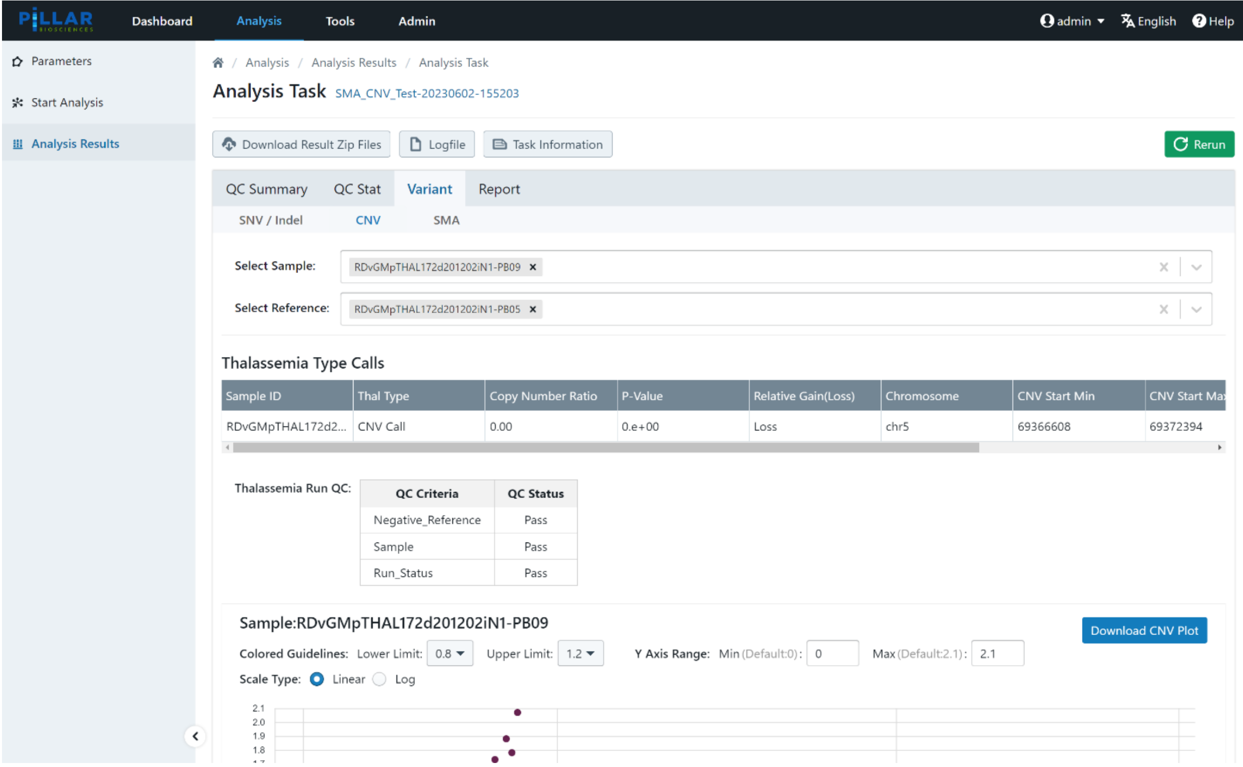


Figure 42 Analysis Results: Thalassemia Type Calls Table

Definition and/or description of result columns reported in the Thalassemia Type Calls table are provided below.

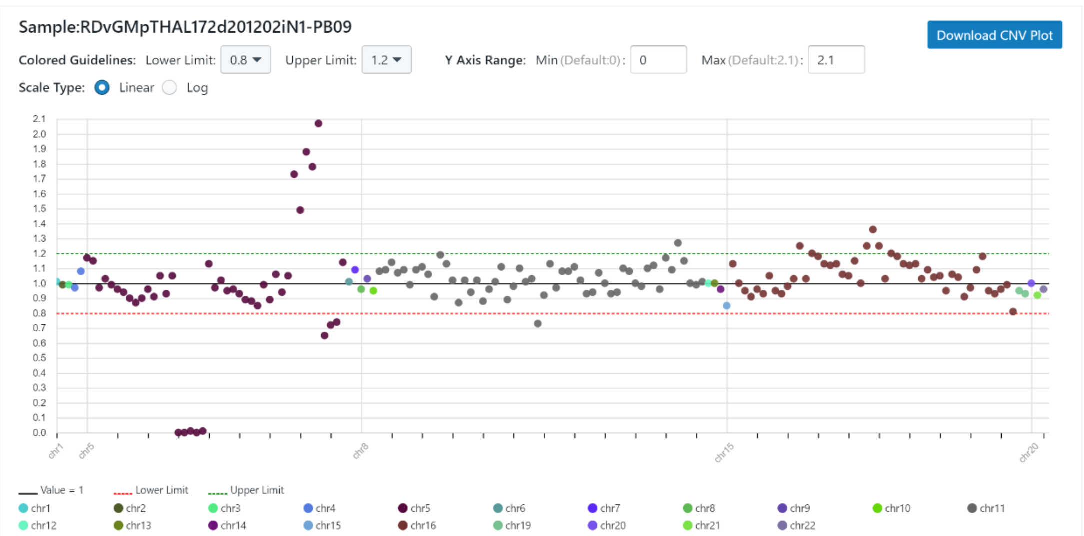
Column Name	Definition/Description
Sample ID	Unique Sample ID for each sample.
Thal Type	The Thalassemia type detected by the "Thal Type Caller" for a sample.

Copy Number Ratio	The ratio of the copy number of called Thalassemia CNV to the copy number (2) of a negative normal sample.
P-Value	P-value of the called Thalassemia CNV.
Relative Gain/Loss	Relative Gain or Loss label of the called CNV segment. If the copy number ratio is more than 1, it is labeled as “Gain”. Otherwise, it is labeled as “Loss”.
Chromosome	The chromosome the Thalassemia type call is located on.
CNV Start Min	The minimum start position of the detected thalassemia.
CNV Start Max	The maximum start position of the detected thalassemia.
CNV End Min	The minimum end position of the detected thalassemia.
CNV End Max	The maximum end position of the detected thalassemia.

Thalassemia Plot

See CNV Plot section in Appendix C: Somatic CNV Caller for a description of CNV/Thalassemia sample plots, and how to save plots as PNG files.

Note that for the Thalassemia plot, the copy number ratios are grouped by chromosome and not by the target genes. The chromosomes can be toggled to show or hide the data points, similar to the CNV plot.



Appendix E: SMA Caller

The SMA analysis is based on the double normalization method. The normalization baseline is calculated from negative reference samples. The SMA Caller calculates the copy number ratios of Exon-07 and Exon-08 amplicons on the SMN1 and SMN2 genes.

SMA Sample Setup

For each SMA analysis run, the user should provide 3-5 (minimum 2) in-run normal (negative) reference samples with similar sample condition and preparation process as the positive samples. If less than 2 negative reference samples are provided, the run will fail.

See CNV Sample Setup section in Appendix C: Somatic CNV Caller for instructions to define normal samples to be used as negative references.

SMA Analysis Setup

5. See CNV Analysis Setup section in Appendix C: Somatic CNV Caller for instructions to setup a SMA Thalassemia analysis.
6. The SMA_RESULTS excel file is output upon completion of the analysis.
7. Detailed SMA call information is in the "SMA Call Report" sheet of the SMA_RESULTS excel file.
8. Each sample's detailed SMA Report can be downloaded as a PDF file in the "Report" section of the results page.
9. The fully normalized copy number ratios can be found in "Fully Normalized" sheet in SMA_RESULTS excel file. Note that the copy number ratio in PiVAT is defined as the copy number ratio of a potentially positive sample to that of the negative reference samples (diploid with 2 copy number).

SMA QC parameters

See CNV QC parameters section in Appendix C: Somatic CNV Caller for a list of user adjustable QC parameters.

PiVAT Output: SMA_RESULTS file

Definition and/or description of result columns reported in SMA_RESULTS file sheets are provided below.

Sheet Tab	Column Name	Definition/Description
SMA Call Report	**Sample ID**	Unique Sample ID for each sample.
	Gene-Exon	Name of the gene/exon pair, or if a control target, name of the control amplicon.
	Location	Genomic coordinates of exon (hg19).
	Copy Number	Copy number of the gene-exon or of the control amplicon
	Copy Number Ratio	The copy number ratio of the gene-exon or control-amplicon to the copy number (2) of a negative normal sample.
SMA Run QC	**QC Criteria**	Indicate which run QC criteria that each row is reporting, including “Negative_Reference”, “Sample” and “Run_Status”.
	QC Status	Whether the QC is passed for each run QC criteria. If run QC is passed, it is labeled as “Pass”. It run QC is failed, it is labeled as “Fail”.
Filtered SMA Samples	**Sample ID**	Unique Sample ID for each sample
	Filter Reason	The reason that the sample is filtered out from the SMA Thalassemia analysis.
	Sample Type	Indicate whether the reported sample is “Sample” or “Negative Reference”. This is pre-defined by the user before starting the SA analysis.
CNV Segment Coverages	This sheet reports the raw segment coverages of each amplicon for all the samples initially added to the SMA analysis. The column headers are Amplicon Names and the row indices are Sample IDs.	
Normalized Coverages	This sheet reports the normalized copy number ratio of each amplicon for the samples that pass the sample QC. Note that the filtered samples are not reported in this sheet. The column headers are Amplicon Names and the row indices are Sample IDs.	

SMA Calls Table

SMA Calls table contains the copy number ratios for the sample(s) selected in **Select Sample.**

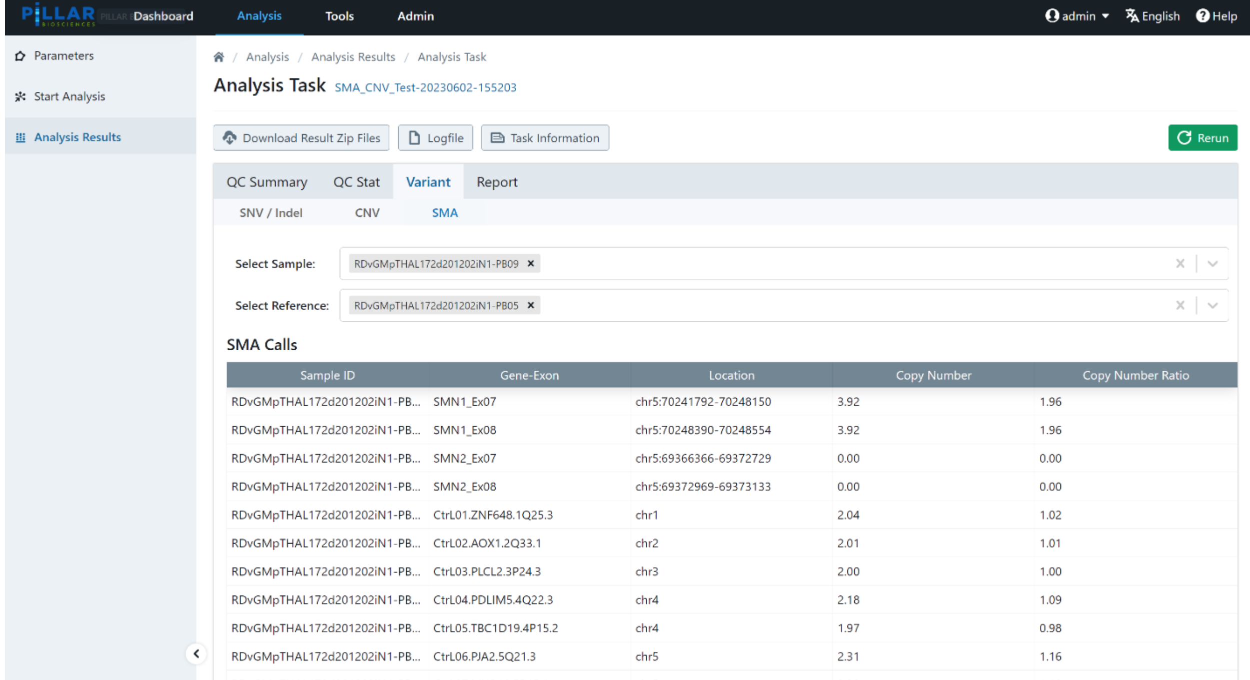


Figure 43 Analysis Results: SMA Calls table

Definition and/or description of result columns reported in the SMA Calls table are provided below.

Column Name	Definition/Description
Sample ID	Unique Sample ID for each sample.
Gene-Exon	Name of the gene/exon pair, or if a control target, name of the control amplicon.
Location	Genomic coordinates of exon (hg19).
Copy Number	Copy number of the gene-exon or of the control amplicon
Copy Number Ratio	The ratio of the copy number of called Thalassemia CNV to the copy number (2) of a negative normal sample.

SMA Plot

A SMA plot for each sample will be located on the Variant > SMA tab below the SMA Calls table and SMA RUN QC table in Analysis Results. Each boxplot represents the copy number ratios of all CNV Normal Samples specified in the run, and the orange data points represent the copy number ratios of the potentially positive sample. The x-axis represents gene-exon labels, and the y-axis represents copy number ratio.

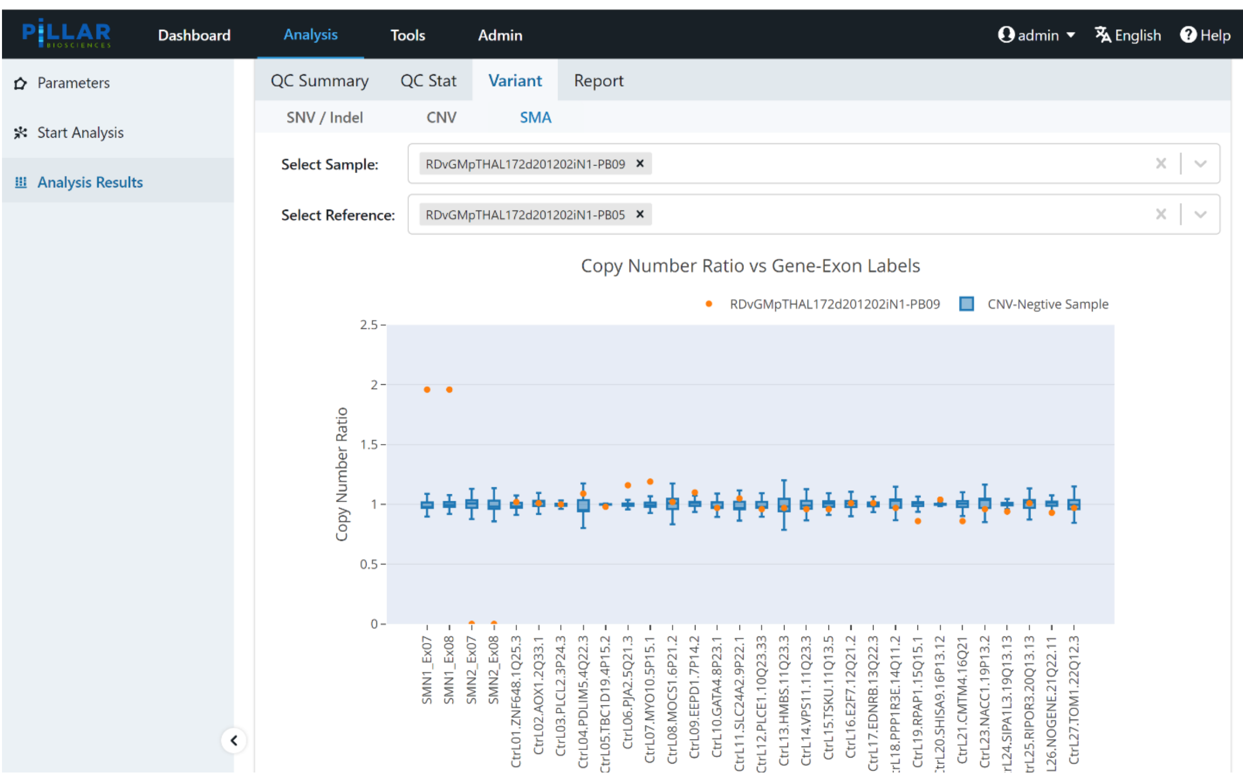


Figure 44 Analysis Results: SMA Sample Box Plot

To save the box plot graph, hover over the graph, and click the "**Download plot as a png**" icon as indicated below.

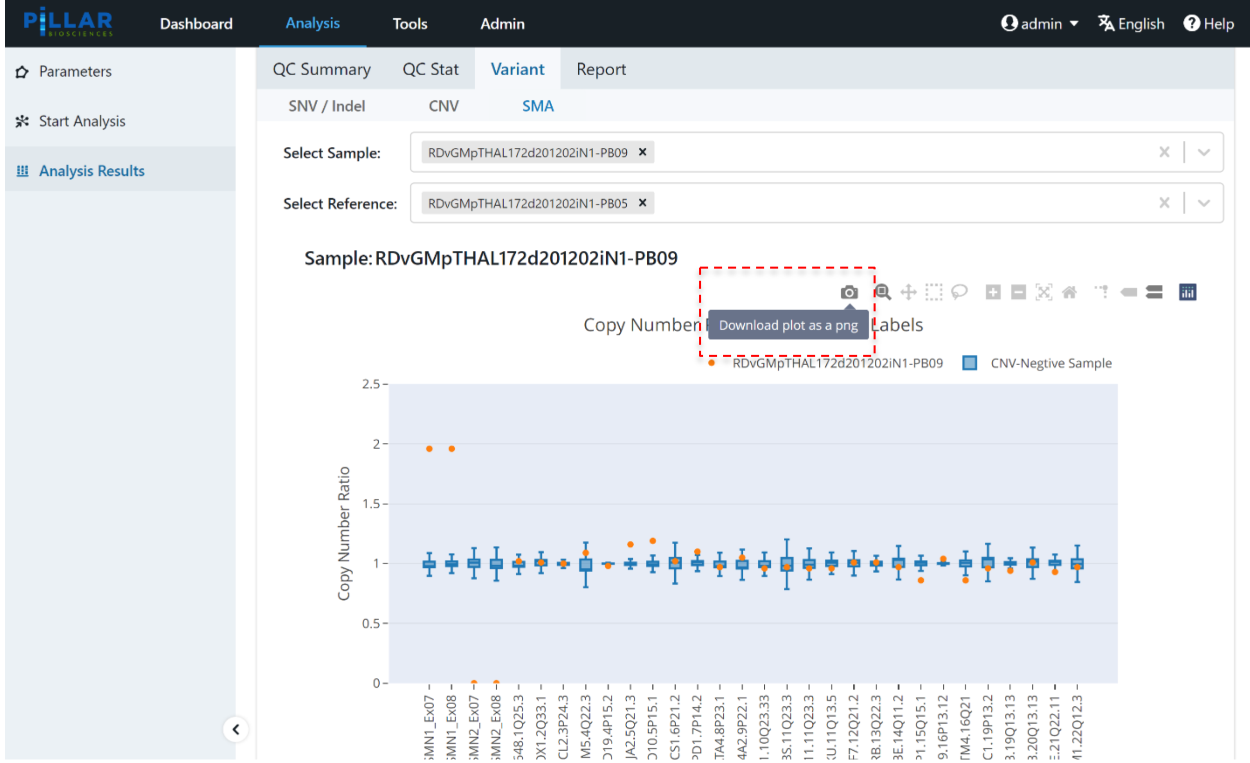


Figure 45 Analysis Results: SMA Sample Box Plot – Saving as PNG

Appendix F: Fusion Caller

The Solid Fusion V2 panel detects common fusion transcripts in a simple, multiplex reaction. The output provides likely fusion transcripts by gene and exon pairs. Only fusions described in the Product Sheet for a given panel will be reported by that panel. Fusions detected, which do not match any described in the Product Sheet, will be filtered from results and recorded in the debug log.

Fusion Sample Setup

Control samples are not required for this panel.

Fusion Analysis Parameters Setup

1. Navigate to ① **Secondary Analysis Parameters** page to access the following list of adjustable parameters and default values in ( ) by tab.

Tab	QC Parameters [default]
Fusion BWA Alignment	**Minimum Distance to Breakpoint (12)** -T parameter input into BWA. Sets a different T value when running bwa which can help getting a shorter alignment as secondary alignment. Decreasing this parameter may increase noise but may help rescue fusion calls for shorter read lengths
Fusion Sample Summary	**Fusion Cutoff Threshold (200)** Minimum supporting reads for a positive fusion call. Usually far below what would be needed for a positive call due to the normalized fusion count cutoff. May need to be adjust if the normalized cutoff is significantly decreased.
	Normalized Fusion Cutoff Threshold (250) Minimum normalized reads for a positive fusion call. NormalizedCount = (Fusion count * Constant) / mapped read count Constant = 100,00 Decreasing this value increases sensitivity but may result in more false positive calls. Increasing this value will decrease sensitivity resulting in possible false negative calls.
	Total Read Count Threshold (100,000) Samples with less total reads than this parameter will fail QC. Decrease if sample input is low. May result in poor quality samples with fusion calls.
	Internal Control Threshold (1,500) The sum of the counts for TBP|HBMS in the IMBALANCE RATIO sheet. Samples with less than 1500 reads for both targets combined will fail QC

PiVAT Output: CUSTOMER_RESULTS file

Definition and/or description of result columns reported in REPORT_FUSION file sheets are provided below.

Sheet Tab	Column/Row Name	Definition/Description
Fusions	**SAMPLE_NAME**	Name of sample
	FusionName	Fusion ID = LeftGene(LeftExon):RightGene(RightExon)
	Count	Number of reads traversing fusion breakpoint
	NormalizedCount	Count / MappedReads * Constant
	HGVS Notation	Fusion ID in HGVS Nomenclature
	Breakpoint	Fusion breakpoint LeftPosition, RightPosition
	LeftStrand	Orientation of left gene. 1 positive strand, -1 negative strand
	RightStrand	Orientation of right gene. 1 positive strand, -1 negative strand
IMBALANCE RATIO	**SAMPLE_NAME**	Name of sample
	Gene	Target gene for balance reads
	Type	Internal control or 3'/5' comparison
	Count	Number of reads on left | Number of reads on right
	Ratio	Imbalance score = (3' balance reads – 5' balance reads)/ reads in control gene
	Detection	"Positive" or "Negative" or "No Call" for evidence of fusion Note: *This is not factored into the Fusions sheet. If positive calls exist it provides support for a fusion event with this gene. The partner should be in the fusion sheet if it was included in the panel's targets.*
	Target_Name	Name of left target | Name of right target
RUN STATS	**Read Count**	The total raw reads in the sample's fastq files
	Assembled	Total count of forward and reverse reads that were paired and merged during paired end assembly. each pair count as 2.
	% Assembled	Percentage of **Assembled** reads out of **Read Count**: • (Assembled / Read Count) * 100
	Mapped Reads	Total number of reads that were successfully aligned by bwa.

Sheet Tab	Column/Row Name	Definition/Description
	Mapping Rate (%)	Percentage of **Mapped Reads** out of **Total Reads Count:** • (Mapped Reads / Read Count) * 100
	Fusion Reads	Reads that have a secondary alignment are considered as fusion reads because they are candidate to a junction point between the primary and secondary alignment. These reads are counted as following: • Assembled reads: if there is a secondary alignment: count as 2 • paired end reads: each read with secondary alignment: count as 1
	FILTERED: Single Side Fusions With Mate Aligned On Different Gene	Unassembled paired end reads where the forward read detected a different junction point than the reverse read.
	FILTERED: Dimers	Reads filtered because fusion was detected to be a dimer.
	FILTERED: Misprimings	Reads filtered because the aligned read was detected to be a mispriming
	Total Valid Fusion Sequences	Total number of reads that cover a junction after filtering.
	Balance Reads	The total number of balance reads.
	FILTERED: Unique Reads Mapped Off Target	All reads that are aligned to untargeted coordinates.
	Total Valid Balance Sequences	Total number of balance reads that are not filtered. Reads are filtered if they are not on targeted regions.
	Total Reads After Filtering	The total reads that were not filtered.
	% Total Unfiltered Reads	Percentage of **Total Unfiltered Reads** out of **Total Reads Count**. (Total Unfiltered Reads / Total Reads Count) * 100
QC FILTERED SAMPLES	**Reason**	Reason sample did not pass QC thresholds

Appendix G: FASTQ File Name Format

A full description of the Illumina FASTQ Name Format can be found under the "Naming" section here:

https://support.illumina.com/help/BaseSpace_OLH_009008/Content/Source/Informatics/BS/NamingConvention_FASTQ-files-swBS.htm

It is not necessary to name FASTQ files following the Illumina naming convention to analyze them through PiVAT.

The following terms should be avoided as sample names: "*common*" and "*logs*". Sample names should not vary by case only.

Sample names are case insensitive when working in Windows or MacOS environments but, case sensitive when working in Linux. This could result in files being hidden, or overwritten.

Do not use any patient identifiable information in any file names.